

MANIPULATION OF THE GROWTH HORMONE IGF-I AXIS IN TYPE ONE  
DIABETES: INSIGHT INTO PHYSIOLOGICAL MECHANISMS AND POTENTIAL  
STRATEGIES FOR THERAPEUTIC INTERVENTION

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## Dedication

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*For Hilda: she would have been very proud.*

*“To talk well and eloquently is a very great art, but an equally great one is to know the  
right moment to stop.”*

*Wolfgang Amadeus Mozart*



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## Abstract

### Background:

Young adults with Type 1 Diabetes have low circulating concentrations of IGF-I with consequent GH hypersecretion. This results in reduced insulin sensitivity and may be implicated in the risk of microvascular complications, particularly nephropathy. Abrogation of these abnormalities may facilitate more normative metabolic and renal physiology and lead to the development of adjunctive therapies for use in this vulnerable population.

### Aims:

- 1) To investigate the effects of specific GH blockade on insulin sensitivity and carbohydrate and lipid metabolism in young adults with Type 1 Diabetes.
- 2) To investigate the effects of restoration of circulating IGF-I concentrations by administration of rhIGF-I complexed with rhIGFBP-3 on GH secretion, overnight insulin requirements, glomerular filtration rate, urinary albumin excretion rate and proteolysis of IGFBP-3.

### Methods:

- 1) In a crossover study, 2 doses (5 and 10mg) of a specific GH antagonist (B2036-PEG, Somavert) were each administered for 3 weeks to 7 subjects, in random order with a 3 week washout period. Euglycaemic clamp techniques incorporating cold isotopes were used to quantify carbohydrate and fat metabolism at baseline and following GH blockade.
- 2) In a double blind placebo controlled crossover study, rhIGF-I/rhIGFBP-3 complex (0.4mg/kg/day) was administered to 6 subjects for 7 days. Overnight GH secretion, insulin requirements, glomerular filtration rate and urinary albumin excretion rate were determined at the end of each treatment period. Proteolysis of IGFBP-3 was assessed using western immunoblotting techniques of plasma and urine before and following administration of rhIGF-I/rhIGFBP-3 complex.

### Results:

- 1) Selective GH blockade using B2036-PEG led to reductions in IGF-I concentrations, reductions in insulin requirement and suppression of lipolysis overnight. During a hyperinsulinaemic euglycaemic clamp, there was no effect on glucose or glycerol turnover.
- 2) Administration of rhIGF-I/rhIGFBP-3 complex led to increases in IGF-I concentrations and suppression of endogenous GH secretion. Overnight insulin requirements were reduced and there was no effect either on glomerular filtration rate or urinary albumin excretion rate. Following rhIGF-I/rhIGFBP-3 complex administration there appeared to be an induction of the proteolysis of IGFBP-3 within 7 hours, with subsequent excretion of proteolysed fragments in the urine.

### Conclusion:

Blockade of the action of excessive GH or suppression of endogenous secretion of GH using novel compounds in Type 1 Diabetes leads to reductions in overnight insulin requirements. Failure to observe effects on other parameters may reflect opposing actions of IGF-I and GH on insulin sensitivity and GFR or the limitations of hyperinsulinaemic clamp methodology. Further evaluation of the clinical relevance of such interventions in larger scale clinical trials would be of interest.

## Declaration

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*I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.*

*This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.*

Rachel Mair Williams

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## List of Abbreviations

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AER	Albumin excretion rate
CSII	Continuous subcutaneous insulin infusion
ERPF	Effective renal plasma flow
FFA	Free fatty acids
GFR	Glomerular filtration rate
GH	Growth Hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
IGFBP-1	Insulin like growth factor binding protein 1
IGFBP-2	Insulin like growth factor binding protein 2
IGFBP-3	Insulin like growth factor binding protein 3
IGF-I	Insulin like growth factor 1
IGF-II	Insulin like growth factor 2
NEFA	Non esterified free fatty acids
NMR	Nuclear magnetic resonance
SK	Somatokine (rhIGF-I/rhIGFBP-3 complex)
S10	Somavert 10mg (Pegvisomant, B2036-PEG)
S5	Somavert 5mg (Pegvisomant, B2036-PEG)
SS	Somatostatin
T1D	Type one diabetes mellitus
T2D	Type two diabetes mellitus

# Chapter 1: INTRODUCTION

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## 1.1. Type One Diabetes Mellitus at Puberty

The management of diabetes during puberty and adolescence presents a number of challenges. The ideal is to achieve optimal glycaemic control in order to minimise the risk of long-term complications by using an insulin regimen that is acceptable to the individual (DCCT, 1994). During adolescence, it is notoriously difficult to achieve adequate glycaemic control and a rise in HbA1c is almost inevitable (Holl, Swift et al. 2003). Attempts to improve control by intensification of insulin therapy may result in unacceptable side effects such as hypoglycaemia (particularly at night) and inappropriate gains in fat mass (DCCT, 1994). Results from large international studies aiming to intensify treatment in this vulnerable population in order to improve glycaemic control and reduce the risk of complications are generally disappointing (Mortensen, Robertson et al. 1998; Holl, Swift et al. 2003).

The management of diabetes during puberty and adolescence is a complex problem, requiring the input of a cohesive multidisciplinary team incorporating a number of essential professionals such as paediatric diabetes specialist nurses, dieticians and psychologists in addition to a paediatrician, with all team members communicating similar goals and expectations to the young person and their family in order to optimise glycaemic control (Hoey, McGee et al. 2006). The adolescent with diabetes is a vulnerable individual, at risk of the complications of diabetes as well as of psychological disturbance. Worryingly, large numbers may become lost to follow up during their transition from the paediatric to the adult clinic, meaning they have no regular input from specialist diabetes teams, and, importantly, no regular screening for the more common complications of diabetes such as retinopathy and microalbuminuria (Bryden, Peveler et al. 2001).

Whilst the effects of *psychological* factors such as rebellion and difficulties with concordance cannot be ignored, there are very real *physiological* influences during puberty, notably the actions of growth hormone and the sex steroids, which can negatively impact on diabetes management during puberty and adolescence (Dunger 1992). Adolescents with Type 1 Diabetes have well described abnormalities of the growth hormone - IGF-I axis, with low circulating concentrations of IGF-I and hypersecretion of growth hormone which leads to insulin resistance, often manifesting as the 'dawn phenomenon' of morning hyperglycaemia (Edge, Dunger et al. 1990; Edge, Matthews et al. 1990). This phenomenon may impede attainment of tight glycaemic control, as increasing evening doses of intermediate or long acting insulin in order to achieve fasting blood sugars within target, may result in inappropriate insulin concentrations in the early hours of the morning with associated hypoglycaemia (Amin, Ross et al. 2003).

Over recent years, the development of insulin analogues, with more attractive pharmacokinetic profiles, has had encouraging effects on rates of overnight hypoglycaemia, with some beneficial effects on glycaemic control in the paediatric population (Mohn, Matyka et al. 1999; Murphy, Keane et al. 2003). However, the pharmacokinetic profiles of the insulin analogues still remain far from ideal and the fluctuation in basal insulin requirements over a 24 hour period can only really be reproduced by continuous subcutaneous insulin infusion (CSII) via a pump (Tamborlane and Press 1984). CSII is used extensively in the USA and Scandinavia and its use in children is gradually becoming more commonplace in the United Kingdom. Introduction of pump therapy leads to improvements in glycaemic control, with reductions in total daily insulin dose and reduced rates of hypoglycaemia (Boland, Grey et al. 1999). They are, however expensive and as their use becomes more widespread, robust confirmation of efficacy within the paediatric population in terms of HbA1c has yet to be achieved (Nahata 2006), although most studies consistently report improvements in quality of life, reductions in total daily insulin dose and reduced rates of hypoglycaemia (Juliussen,



Graue et al. 2006). Within England and Wales, the use of CSII in children is subject to guidance by the National Institute of Health and Clinical Excellence (NICE) which recommends them only in children if multiple daily injection therapy is deemed unsuitable (children <11yr) or if adequate glycaemic control is unacceptable (HbA1c>8.5%), or in the face of severe and disabling hypoglycaemia.

In the adolescent with diabetes, although insulin deficiency is clearly the primary endocrinopathy, the influence of other hormonal perturbations, particularly those of the growth hormone IGF-I axis are also important and the abnormalities of this axis will be discussed in detail later in this chapter. Growth hormone hypersecretion leads to increased insulin resistance and may independently contribute to the risk of development of microvascular complications, in particular, nephropathy. More detailed understanding of the dysregulation of this axis and its physiological effects may permit the development of alternative strategies for therapy, in an attempt to mimic more normative physiology. Such strategies could be used in conjunction with optimal insulin replacement therapy in the vulnerable adolescent population in order to reduce the risk of complications by means independent of glycaemic control. In addition, manipulation of this axis in the young person with Type 1 Diabetes gives a unique opportunity for furthering our understanding of the physiological and metabolic effects of growth hormone and its dependent peptides.

## 1.2. Growth Hormone

### 1.2.1 *Regulation of Secretion*

Growth hormone is a non-glycosylated, single chain, 191 amino acid, 22 -kDa protein which is synthesised and secreted mainly by somatotrophs located in the lateral segments of the anterior pituitary gland (Kopchick, Parkinson et al. 2002). Growth hormone is released in a pulsatile fashion due to the interaction of 2 hypothalamic factors; Growth hormone releasing hormone (GHRH) and Somatostatin, which stimulate and inhibit its release respectively (Figure 1) (Muller, Locatelli et al. 1999). GHRH and



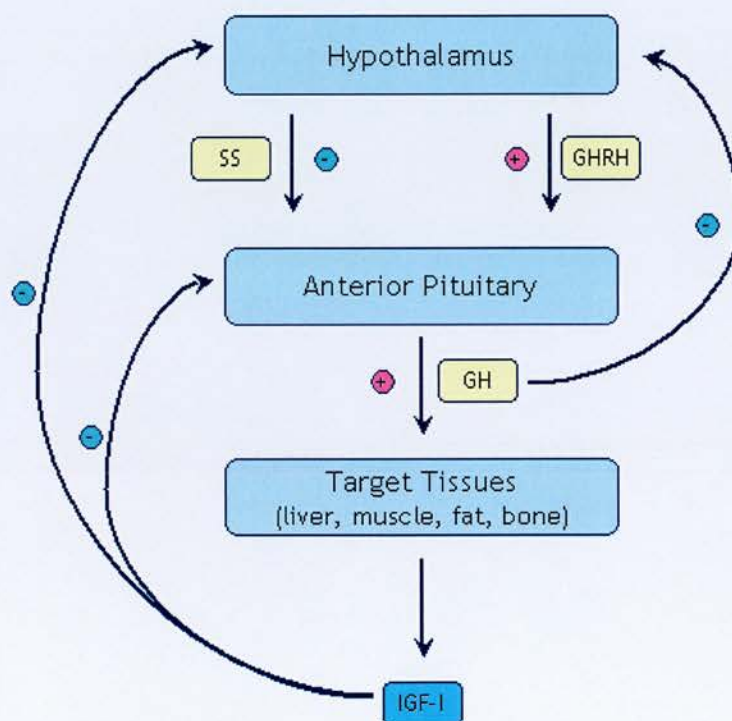
Somatostatin are themselves released in pulses and the integration of these, together with the influence of numerous other growth hormone secretagogues, results in the classical pulsatility of endogenous growth hormone release, reviewed comprehensively in Physiological Reviews, 1999 (Muller, Locatelli et al. 1999). Patterns of GHRH and Somatostatin release are themselves determined by the complex interaction of neurotransmitters, neuropeptides and opioids, and so the final secretory pattern of growth hormone is the end result of an elegant and complex interaction between numerous regulators (Muller, Locatelli et al. 1999).

At the cellular level, the mechanism of growth hormone release by somatotrophs is dependent upon a cyclic AMP (cAMP) mediated rise in intracellular calcium concentration with subsequent induction of the transcription of pituitary specific transcription factors such as *pit-1* (Giustina and Veldhuis 1998). GHRH leads to a rise, and Somatostatin to a fall, in intracellular calcium concentration and thus they have opposing effects on growth hormone secretion (Melmed and Kleinberg 2003).

Under normal circumstances, maximal growth hormone secretion occurs in the early part of the night during slow wave sleep, but also forms part of the physiological response to a variety of stresses such as prolonged fasting, exercise, trauma, hypovolaemia and hypoglycaemia (Isidori, Fraiolo et al. 1976; Sutton and Lazarus 1976). The quantity of growth hormone secreted declines with age and is suppressed during chronic illness, depression or psychosocial deprivation in children (Melmed and Kleinberg 2003). In addition to regulation by the hypothalamic factors described above, growth hormone secretion is also subject to classical negative feedback inhibition; with growth hormone inhibiting the release of GHRH by the hypothalamus and insulin like growth factor I (IGF-I) inhibiting both GHRH release at the level of the hypothalamus (long loop) and growth hormone release by the pituitary gland (short loop) (Figure 1).

### 1.2.2 Receptor Binding and Downstream Signalling

Growth hormone exerts its effects at target tissues via its receptor (GHR), which consists of an extracellular domain (for ligand binding) linked to a signalling region located on the cytoplasmic side of the cell membrane by a single membrane spanning region (Leung, Spencer et al. 1987). The GHR is ubiquitously expressed in all tissues in humans, however the highest levels of expression occur in bone (at the growth plate), liver, fat and muscle (Kopchick, Parkinson et al. 2002). Binding to the receptors in these tissues effects the growth promoting and metabolic actions of growth hormone which will be described in more detail later in this chapter.

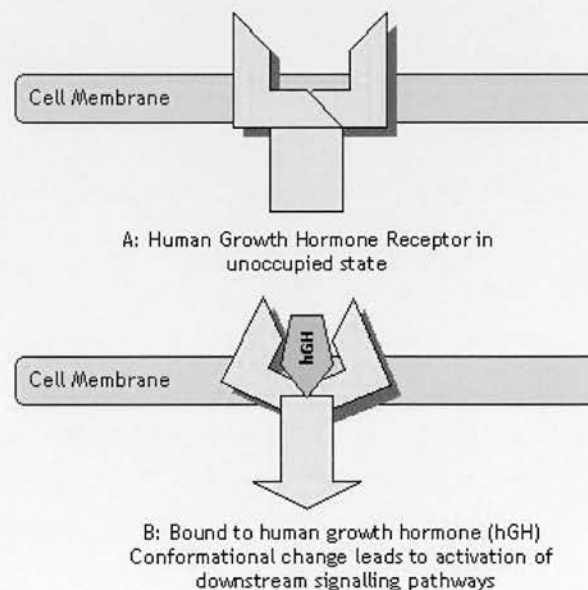


**Figure 1: Regulation of Growth Hormone Secretion**

Schematic representation of the central mechanisms (both negative and positive) of the control of endogenous growth hormone secretion. [GH: Growth Hormone, GHRH: GH Releasing Hormone, SS: Somatostatin, IGF-I: Insulin-like growth factor-I].

Each growth hormone molecule contains 2 receptor binding sites, which allow binding to a GHR dimer, comprising 2 GHR molecules closely approximated on the cell membrane (Gent, Van Den Eijnden et al. 2003). Binding of a single growth hormone molecule to the receptor dimer, effects a conformational change in the intracellular portion of the receptor (Figure 2), and results in activation of an intracellular kinase; Janus Kinase 2 (JAK2) (Carter-Su, Schwartz et al. 1996). Subsequently, phosphorylation leads to activation of intracellular transcription molecules, (specifically the ‘signal-transducing activators of transcription’ proteins (STATs)), which are then translocated to the nucleus where they bind to DNA and regulate transcription of growth hormone specific genes (Xu, Wang et al. 1996). GHR molecules are subsequently internalised within clathrin-coated vesicles and transported to the lysosomes where they are degraded and recycled (Kopchick, Parkinson et al. 2002).

At the cellular level, the actions of growth hormone are terminated by the ‘suppressor of cytokine signalling’, or SOCS proteins which inhibit the JAK2 facilitated translocation of STAT proteins to the nucleus, this disrupting GH signalling (Starr and Hilton 1998).



**Figure 2: Human Growth Hormone Receptor Signalling**

Schematic representation of Human Growth Hormone Receptor activation and signalling

A: Inactive Form B: Bound to human growth hormone.

In addition to activation of its own signalling cascade, the binding of growth hormone to its receptor also affects several other intracellular signalling pathways, notably that of insulin; leading to phosphorylation of both insulin receptor subunits 1 and 2 (IRS-1 and IRS-2) (Carter-Su, Schwartz et al. 1996). Such subtle and intricate interaction of the intracellular signalling pathways of different molecules is an example of the exquisite regulation of metabolism at a molecular level and is, as yet, far from fully understood (Carter-Su, Schwartz et al. 1996; Dominici, Argentino et al. 2005).

### 1.3. The Metabolic Effects of Growth Hormone

In addition to regulating growth at the growth plate, both directly and also indirectly via the generation of IGF-I, growth hormone has a number of effects on metabolism. It has anabolic effects on muscle; enhancing protein synthesis, it stimulates lipolysis leading to alterations in patterns of fat deposition and also affects glucose metabolism, leading to reductions in insulin sensitivity (Melmed and Kleinberg 2003). The effects of growth hormone on fat turnover and on insulin sensitivity will now be discussed in further detail.

#### 1.3.1 *Fat Metabolism*

Growth hormone has well described effects on lipid metabolism; its predominant effect being the stimulation of lipolysis that leads to release of non-esterified free fatty acids (NEFA) from adipocytes (Piatti, Monti et al. 1999). Moller and colleagues performed detailed physiological studies in 6 healthy volunteers, each of whom was studied on 2 occasions, once following an infusion of growth hormone (20mU/kg/min for 4 hours) and once following an infusion of 0.9% sodium chloride (Moller, Jorgensen et al. 1990). After the infusion of growth hormone, there was evidence of a rapid induction of lipolysis, in conjunction with a 40 to 50% reduction in glucose utilisation in the forearm. There was a rapid rise in concentrations of free fatty acids and  $\beta$ -hydroxybutyrate which peaked after 2 to 3 hours (Moller, Jorgensen et al. 1990). The same group carried out detailed physiological studies of the effects of different doses (70, 140 and 350

micrograms administered as an intravenous pulse) of growth hormone on parameters of glucose and lipid metabolism, reporting dose-dependent effects on lipolysis, but not on glucose metabolism (Moller, Schmitz et al. 1992).

Adults with growth hormone deficiency have marked aberrations of serum lipid parameters; increased cholesterol, decreased HDL cholesterol, a decreased HDL: LDL ratio and increased triglycerides, a cluster of parameters that confers an increased risk of cardiovascular disease (de Boer, Blok et al. 1995). Growth hormone deficiency in adults manifests as a recognisable syndrome that includes impaired quality of life, body composition changes including increased fat mass with altered distribution and increased risk of cardiovascular disease and stroke (Salomon, Cuneo et al. 1989). In contrast, in the hypersomatotropic state of acromegaly there is insulin resistance as demonstrated by increased fasting glucose, insulin and C-peptide concentrations combined with a 50% greater rate of lipid oxidation than control subjects, differences which largely disappear following pituitary surgery with subsequent appropriate replacement of anterior pituitary hormones (Moller, Schmitz et al. 1992).

### *1.3.2 Insulin Sensitivity*

The predominant insulin responsive tissues in man are liver, muscle and fat (Melmed and Kleinberg 2003). In liver, insulin suppresses endogenous hepatic glucose production from the breakdown of hepatic glycogen and stimulates glycogen synthesis. In muscle, insulin stimulates the uptake of glucose for peripheral utilisation and the synthesis of muscle glycogen and in fat; hormone sensitive lipase is exquisitely sensitive to insulin, with relatively low concentrations of insulin leading to almost complete suppression of lipolysis and induction of lipogenesis. Following a meal, insulin release into the portal circulation in response to enteral feeding, signals the switch from the fasting to the fed state.

The quantification and description of insulin sensitivity is complicated by the use of many different methodologies, however the hyperinsulinaemic euglycaemic clamp



technique incorporating the use of cold isotopes allows the estimation of contribution of both hepatic and peripheral (predominantly muscle) insulin sensitivity and is considered to be the gold standard methodology (DeFronzo, Tobin et al. 1979). Hepatic and peripheral insulin sensitivity can be considered separately; hepatic insulin sensitivity reflecting the amount of insulin required to suppress hepatic glucose production during a fast (for example overnight) and peripheral insulin sensitivity reflecting the rate of insulin stimulated glucose uptake by muscle (for example following a meal) (DeFronzo, Tobin et al. 1979).

Growth hormone has been known to have effects on glucose homeostasis since the 1930s when Houssay described a reduction in the blood sugars of dogs with experimental diabetes following hypophysectomy (Houssay and Biasotti 1930). Growth hormone was subsequently isolated from pituitary extracts and confirmed as the diabetogenic agent. Since then the details of the cellular and molecular mechanisms by which growth hormone influences insulin action have been extensively (but by no means exhaustively) characterised.

Growth hormone has effects both on hepatic and peripheral insulin sensitivity in humans. In healthy volunteers, approximately 5 hours after administration of a Growth hormone bolus, there is a right shift in the dose response curve of both hepatic glucose production and peripheral glucose utilisation to insulin (Rizza, Mandarino et al. 1982), implying that growth hormone has effects both on hepatic and peripheral insulin sensitivity. The reduction in insulin sensitivity following growth hormone administration may be mediated via effects on the insulin-signalling pathway. There are common elements to the signalling mechanisms of both the insulin and growth hormone receptors; notably the kinase, JAK 2 (Carter-Su, Schwartz et al. 1996). Interaction at the intracellular level following activation of either or both receptors is likely to provide a mechanism by which growth hormone and insulin can influence each other's actions,

however, the exact nature of this subtle interaction remains to be determined (Carter-Su, Schwartz et al. 1996).

Growth hormone may also influence insulin sensitivity indirectly, by its effects on lipolysis. Growth hormone stimulates lipolysis, leading to elevations in circulating concentrations of free fatty acids (FFA) (Piatti, Monti et al. 1999). FFAs have long been known to correlate inversely with insulin sensitivity. In the 1960s, Sir Philip Randle proposed the glucose fatty-acid cycle, whereby fatty acids induce insulin resistance in muscle, by the inhibition of phosphofructokinase and subsequent inhibition of cellular glucose uptake (Randle, Garland et al. 1963). More recently, the development of nuclear magnetic resonance (NMR) spectroscopy has permitted quantification of intra- and extra-myocellular triglycerides and confirmed that an increase in plasma fatty acid concentration leads to reductions in glucose uptake by the myocyte (Shulman 2000). This is likely to be secondary to an accumulation of intramyocellular fatty acyl coA in conditions of elevated plasma fatty acids, with subsequent effects on downstream signalling from the insulin receptor, namely reductions in IRS-1 associated PI3 kinase activity culminating in reductions in glucose transport into the muscle (Dresner, Laurent et al. 1999; Savage, Petersen et al. 2007). The understanding of the cellular mechanisms of free fatty acid mediated effects on insulin sensitivity at the liver are less clear, but free fatty acids appear to stimulate gluconeogenesis, at least at supra-physiological concentrations in subjects with T2D, but not following an overnight fast (Boden, Chen et al. 2001).

### *1.3.3 The Effects of Growth Hormone on Renal Physiology*

It has been known since the 1950's that growth hormone mediates glomerular haemodynamics, when Ikkos reported increased effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) in a group of patients with acromegaly (Ikkos, Ljunggren et al. 1956). Increased urinary albumin excretion is a characteristic feature of hypersomatotropic states such as acromegaly, while patients with growth hormone

deficiency have reduced GFR and ERPF (Hoogenberg, Sluiter et al. 1993). In humans, exogenous administration of recombinant human growth hormone leads to increases in both GFR and ERPF (Hirschberg, Rabb et al. 1989) and administration of somatostatin analogues, (which block growth hormone secretion), to patients with acromegaly leads to reductions in GFR, ERPF and urinary albumin excretion rates (Dullaart, Meijer et al. 1992; Manelli, Bossoni et al. 2000).

It is unclear whether growth hormone's effects on renal physiology are due to a direct effect on the kidney, or mediated via increases either in circulating or intra-renal concentrations either of IGF-I or epidermal growth factor (EGF) (Flyvbjerg 2000). Very detailed studies of the expression of growth hormone and its dependent peptides have been performed in rodents (Flyvbjerg 2000). However, the information pertaining to the human kidney, particularly in healthy humans is, (for reasons which are perhaps obvious!) very limited, but the available data indicate that there may be important differences between species. For example, in the rat nephron, expression of IGF-I, but not IGF-II has been demonstrated, in contrast to the human where there is no detectable evidence of IGF-I transcription (in the form of IGF-I mRNA), but IGF-II mRNA can be detected, predominantly localised to the perivascular regions (Chin and Bondy 1992). Caution must therefore be exercised when extrapolating the findings from animal work to humans, both in healthy animals and in experimental models of diseases such as diabetes.

Most available evidence from healthy humans supports the hypothesis that GH exerts its effects on renal haemodynamics secondary to an increase in circulating IGF-I, with effects on GFR being delayed and only becoming apparent after 24hrs, concomitant with a rise in IGF-I. i.e. growth hormone appears to affect GFR chronically rather than acutely, which may reflect the temporal delay in the rise in circulating IGF-I concentrations following commencement of exogenous growth hormone administration (Hirschberg, Rabb et al. 1989).



The expression of IGF binding protein-like proteins on the membrane of renal tubular cells implies that, in addition to straightforward re-absorption and degradation by tubular cells as a means of clearing IGF-I, that IGF-I, along with other growth factors may exert a local effect within the tubular cells but as yet the details of these have not been characterised (Flyvbjerg, Landau et al. 1995).

#### 1.4. Insulin-Like Growth Factor – I

##### 1.4.1 *Historical Background*

The insulin-like growth factors were first discovered in 1957; when Salmon and Daughaday demonstrated that radio-labelled sulphur could be incorporated into chondrocytes in vitro (Salmon and Daughaday 1957). They isolated two peptides of molecular weight approximately 7 –kD and named them somatomedin 1 and 2. Further work led to characterisation of their insulin-like actions when Froesch reported that the addition of anti-insulin antibodies to serum did not completely ameliorate insulin like activity (Froesch, Burgi et al. 1963). By 1972, the somatomedins were known to have growth hormone dependent serum concentrations and stimulatory effects on DNA synthesis and cell proliferation (Daughaday, Hall et al. 1972). Their metabolic actions, in particular their insulin-like actions, resulted in them being re-named insulin-like growth factors 1 and 2; IGF-I and IGF-II (Rinderknecht and Humbel 1976).

##### 1.4.2 *Structure and Regulation of Secretion*

The IGF-I gene is located on the long arm of chromosome 12 and encodes a basic peptide 70 amino acids in length (Brissenden, Ullrich et al. 1984; Tricoli, Rall et al. 1984). The translated gene product, human IGF-I, has a molecular weight of approximately 7 -kDa (Brissenden, Ullrich et al. 1984; Tricoli, Rall et al. 1984). It is structurally very similar both to insulin and to IGF-II, sharing approximately 50 and 61% amino acid homology respectively with these family members (Rinderknecht and Humbel 1978). In common with insulin, its secondary structure comprises A and B chains held together by disulphide bonds. In addition, there is a C-peptide region of 12

amino acids, which connects the A, and B chains, but despite there being a similar region in the pro-insulin molecule, this region of IGF-I bears no homology to insulin (Rinderknecht and Humbel 1978).

The secretion of IGF-I is predominantly growth hormone dependent and evidence of IGF-I transcription can be demonstrated approximately 30 minutes following injection of growth hormone into rats (Mathews, Norstedt et al. 1986). In humans, the majority of circulating IGF-I is synthesised and secreted by the liver, following the binding of growth hormone to its hepatic growth hormone receptor (Schwander, Hauri et al. 1983). The expression of the hepatic growth hormone receptor is exquisitely sensitive to concentrations of insulin within the portal circulation; thus low levels of portal insulin lead to reduced expression of the hepatic growth hormone receptor and relative hepatic resistance to growth hormone, which, in turn leads to reduced concentrations of circulating IGF-I (Clayton, Holly et al. 1994). In addition to the liver, many other tissues including muscle, kidney and the growth plate, express the growth hormone receptor, and are capable of local IGF-I transcription and translation (D'Ercole, Stiles et al. 1984). In humans, several authors have described an increase in the generation of IGF-I mRNA in adipocytes following growth hormone stimulation in vitro (Doglio, Dani et al. 1987; Gaskins, Kim et al. 1990).

#### *1.4.3 Receptor binding and downstream signalling*

IGF-I will bind to several receptors, including both the type one and type two IGF receptors and the insulin receptor, in addition to hybrid and atypical IGF receptors (Soos, Field et al. 1993). The type one IGF receptor is expressed widely in humans, with liver being the notable exception (Caro, Poulos et al. 1988). It shares approximately 70% homology with the insulin receptor and binds both IGF-I and IGF-II with great affinity (Massague and Czech 1982; Ullrich, Gray et al. 1986). The type 1 IGF receptor will also bind insulin, but with considerably lower affinity (about 100 times) (Steele-Perkins, Turner et al. 1988). In common with the insulin receptor, the type one IGF

receptor comprises 4 subunits; 2 ligand binding (alpha) subunits which span the membrane and 2 predominantly intracellular beta subunits which undergo tyrosine kinase mediated autophosphorylation leading to activation of the intracellular signalling cascade, which includes insulin receptor substrate 1 (IRS-1) (Siddle, Urso et al. 2001). The intracellular signalling pathways of insulin and IGF-I have many common elements with both ultimately influencing gene expression (Siddle, Urso et al. 2001). Effects on growth and metabolism are presumably exerted at this level. Effects on metabolism are mainly effected via the PI3 kinase pathway, with effects on growth and differentiation being effected via activation of the MAP kinase pathway (Shepherd, Nave et al. 1996). It is currently not clear how cells distinguish between the 2 ligands (IGF-I and insulin) in order for them to exert differential effects, given the seemingly identical signalling pathways of the type one IGF, and insulin receptors (Siddle, Urso et al. 2001).

In contrast, the type 2 IGF receptor bears no similarity either to the type one IGF receptor, or to the insulin receptor and binds IGF-II with high affinity, binding IGF-I only weakly. It is identical to the mannose 6 phosphate receptor, which is responsible for the intracellular targeting of lysosomal proteins (Morgan, Edman et al. 1987; MacDonald, Pfeffer et al. 1988). It has been suggested that IGF-II may be integral to the targeting of intracellular proteins in some way, or, alternatively that the type 2 IGF receptor may simply be a way of clearing IGF-II from the circulation (Meyer, Eskelinen et al. 2001).

Hybrid insulin/ type one IGF receptors have also been demonstrated in mammalian tissues (Soos and Siddle 1989). Hybrid receptors are made up of one insulin receptor dimer and one type 1 IGF receptor dimer, which are linked to form a tetramer (Feltz, Swanson et al. 1988). Hybrid receptors bind both IGF-I and insulin with high affinity but their physiological significance remains uncertain (Soos, Field et al. 1993).

#### 1.4.4 *Biological Effects of IGF-I*

Given the almost universal expression of IGF-I, it has been difficult to distil out the relative effects of circulating IGF-I concentrations as opposed to paracrine effects within the target tissues secondary to local expression. The study of the phenotypes of a number of animal models with selective mutations of individual components of either IGF-I or its related peptides has given a degree of insight into these differential effects but the picture is by no means complete.

##### 1.4.4.i Growth

The phenotypes of mice null for the IGF-I gene, or the type 1 IGF receptor include severe intrauterine growth retardation and early postnatal death (Baker, Liu et al. 1993). The fragility of these null species has made more detailed postnatal physiological studies impossible but more recently, sophisticated molecular techniques have permitted the development of a mouse model which has selective reduction in hepatic IGF-I expression with unaffected expression in all other tissues (the **Liver IGF-I Deficient** or **LID mouse**) (Yakar, Liu et al. 1999). These animals have low circulating concentrations of IGF-I, with increased growth hormone secretion, and have normal linear growth, although with reduced bone mineral density and reductions in growth plate proliferation (Yakar, Liu et al. 1999). Animals with a null mutation for the acid labile subunit (ALS) of the ternary complex have a similar phenotype with reduced circulating IGF-I concentrations but no increase in growth hormone secretion (Haluzik, Yakar et al. 2003). It is only when animals have a double knockout for both ALS and the hepatic IGF-I gene, resulting in further decreases in circulating IGF-I concentrations, that postnatal linear growth is compromised (Haluzik, Yakar et al. 2003).

The human models of mutations in IGF-I and related peptides are extremely rare and do not exhibit the tissue specificity afforded by the mouse models but they are in themselves extremely informative. In the human, functional IGF-I generation appears vital for **both** intrauterine and postnatal growth, with mutations either in the growth

hormone receptor (Laron Syndrome), the IGF-I gene or the type 1 IGF receptor resulting in IUGR and poor postnatal growth (Laron, Kowadlo-Silbergeld et al. 1980; Walenkamp, Karperien et al. 2005; Walenkamp and Wit 2006). The copy number of the IGF-I gene also has implications for altered growth potential; a child with a single copy of the IGF-I gene has been reported with IUGR, postnatal growth failure and hypoglycaemia, in contrast to a child who was born large for gestational age who subsequently demonstrated rapid postnatal growth who was found to have 3 copies of the IGF-I gene (Woods, Camacho-Hubner et al. 1996; Okubo, Siddle et al. 2003).

#### 1.4.4.ii Metabolism

As its name suggests, IGF-I has insulin-like actions; following receptor binding there is activation of its intracellular signalling cascade, which is almost identical to that of insulin. IGF-I has direct actions on glucose metabolism and causes hypoglycaemia approximately 30min after injection when administered to healthy volunteers (Guler, Zapf et al. 1987). Detailed studies in healthy humans suggest that IGF-I administration acutely suppresses both endogenous hepatic glucose production, and stimulates peripheral glucose utilisation during a hyperinsulinaemic clamp, but has no effects on lipolysis (Russell-Jones, Bates et al. 1995). Detailed studies of glucose metabolism in the LID mouse suggest that these animals have reduced insulin sensitivity at the muscle, but that their hepatic insulin sensitivity is unaffected indicating that IGF-I generated by the liver acts peripherally at the muscle in an endocrine fashion to enhance insulin sensitivity, but that there is no direct paracrine effect of IGF-I on insulin sensitivity at the liver at least in this animal model (Yakar, Liu et al. 2001).

IGF-I also exerts anabolic effects on protein metabolism, and leads to reductions in protein turnover and increases in lean body mass when administered to rats for 21 days (Frick, Oscarsson et al. 2000). The anabolic effects of IGF-I have been confirmed in humans using sophisticated physiological studies of amino acid turnover and have

shown to be independent of and of greater magnitude than, the actions of insulin (Fryburg, Jahn et al. 1995).

In hypophysectomised rats, administration of IGF-I for 21 days leads to a reduction in glucose uptake by adipocytes and an increase in glucose uptake by skeletal muscle cells, along with a concomitant reduction in plasma insulin concentrations (Frick, Oscarsson et al. 2000). The differences in glucose uptake between fat and muscle may be secondary to the reduction in insulin concentrations, leading to reduced glucose uptake by the exquisitely insulin sensitive adipocyte (Frick, Oscarsson et al. 2000). Currently, although new details regarding the intracellular signalling of growth hormone, insulin and IGF-I are being elucidated and reported, the finer details of intricate intracellular cross-talk and subtle regulation between the 3 systems remains far from completely understood.

#### 1.4.4.iii Renal Physiology

As previously discussed, subjects with conditions of growth hormone excess such as acromegaly have elevated glomerular filtration rate and effective renal plasma flow, in conjunction with elevations in plasma IGF-I concentrations. Most authors concur that the observed effects on renal haemodynamics following growth hormone treatment are secondary to elevations in plasma IGF-I concentrations, both in healthy humans and in rodents (Hirschberg and Kopple 1989; Hirschberg, Rabb et al. 1989; Hirschberg, Brunori et al. 1993).

Administration of IGF-I to healthy volunteers, and to patients with chronic renal impairment leads to increases in GFR and ERPF and so it would seem that circulating concentrations of IGF-I are able to influence renal haemodynamics directly (Hirschberg and Kopple 1989; Hirschberg, Brunori et al. 1993).



### 1.5. The IGF Binding Proteins

The pool of IGF-I within the circulation is a dynamic resource, comprising 'free' or bioactive IGF-I and IGF-I bound to binding proteins. Only a small proportion of IGF circulates in its unbound or 'free' form, with the majority bound to one of the binding proteins which serve to increase the half-life, facilitate transport and regulate the bioactivity and tissue delivery of IGF peptides.

The IGF binding proteins are a family of soluble proteins which bind both IGF-I and IGF-II with high affinity. So far 6 have been characterised to varying extents in humans and there is around 80% sequence homology between species, particularly in the ligand binding regions which are rich in cysteine residues (Firth and Baxter 2002). The pool of IGF-I within the circulation is a dynamic resource, comprising 'free' or bioactive IGF-I and IGF-I bound to binding proteins. All 6 binding proteins bind both IGF-I and IGF-II with high affinity, with the possible exception of IGFBP-6, which has a much greater affinity for IGF-II (Clemmons 1993). IGFBP-1 and IGFBP-3 are the most comprehensively described of the IGFBPs in humans and will now be discussed in further detail.

#### *1.5.1 IGF Binding Protein 1*

IGFBP-1 is a 30 -kDa non-glycosylated protein that binds both IGF-I and IGF-II with high affinity. Binding to IGFBP-1 has an inhibitory effect on IGF-I action in vitro and thus regulation of production of IGFBP-1 may serve to regulate the acute regulation of bioavailable IGF-I (Clemmons, Busby et al. 1995). Acutely, IGFBP-1 production is exquisitely sensitive to circulating insulin concentrations, which inhibits its production. IGFBP-1 may function as an acute regulator of IGF-I bioavailability, with suppression of IGFBP-1 production by insulin (for example following a meal) leading to an increase in bioavailable IGF-I to potentiate glucose uptake by target tissues (Clemmons, Busby et al. 1995).

### 1.5.2 IGF Binding Protein 3

IGFBP-3 is the most abundant IGFBP in humans and binds approximately 75% of circulating IGF-I (Martin and Baxter 1986). IGFBP-3 is a 266 amino acid protein, which undergoes variable amounts of post-translational modification in the form of N-linked glycosylation at 3 potential sites, leading to a molecular weight which can vary between 30 and 56-kDa (Firth and Baxter 1995). IGFBP-3 can bind IGF-I alone, but the 2 predominantly coexist in a 150-kDa ternary complex with the acid labile subunit (ALS), an 88-kDa glycoprotein (Baxter and Martin 1989). Incorporation of IGF-I into the ternary complex extends its half-life from minutes to approximately 15 hours, thus creating a relatively stable pool of IGF-I within the circulation (Baxter and Martin 1989). The synthesis of both IGFBP-3 and ALS appear to be predominantly regulated by growth hormone and concentrations tend to be low in patients with hypopituitarism and Laron dwarfism with some authors advocating its use as a clinical marker of growth hormone deficient states (Thalange, Price et al. 1996). Administration of either IGF-I or growth hormone to growth hormone deficient mice results in an increase in IGFBP-3 concentrations so it would seem that growth hormone may stimulate the hepatic production of IGFBP-3 either via IGF-I, or that IGF-I may act independently to induce transcription and translation of the IGFBP-3 gene (Camacho-Hubner, Clemmons et al. 1991).

IGFBP-3 is also subject to degradation by protease activity which provides a further level of regulation of IGFBP-3 concentrations and hence IGF-I bioavailability (Baxter, Suikkari et al. 1993). IGFBP-3 protease activity was initially described in the serum of pregnant women; proteolysis of IGFBP-3 leads to the formation of fragments that bind IGF-I with reduced affinity, thus increasing the bioavailability of IGF-I (Baxter, Suikkari et al. 1993). IGFBP-3 proteases have not yet been fully characterised but are thought to be serine, or metallo-proteases (Bunn and Fowlkes 2003; Fowlkes, Serra et al. 2004). Increased activity of IGFBP-3 proteases has been described in type one diabetes and has also been associated with certain malignancies and catabolic states (Lassarre, Duron et



al. 2001). For example, prostate specific antigen stimulates the proteolysis of IGFBP-3, leading to a reduced binding affinity for IGF-I. It is suggested that this leads to increased bioavailability of IGF-I, with potential enhancement of its biological effects (Bunn and Fowlkes 2003).

Thus regulation of IGFBP-3 synthesis and of its subsequent proteolysis under certain conditions permits a dynamic regulation of bioavailable IGF-I but the exact nature or significance of the mechanisms which induce and regulate this remain uncertain.

### *1.5.3 Other Members of the IGFBP Super family*

Whilst IGFBP-1 and IGFBP-3 are the best characterised of the IGF binding proteins, there are other binding proteins which have been isolated as a result of their strong interaction with ligand (i.e. IGF-I and IGF-II). However, it has more recently become apparent that the IGFBPs are part of a super family of proteins sharing sequence homology and include a number of so-called IGFBP related peptides (IGFBPrps) which have been characterised from biological systems distinct than the IGF-I axis, and retrospectively been discovered to have significant sequence homology to the IGF binding proteins, particularly within the N terminal cysteine-rich region, comprehensively reviewed by Hwa and Rosenfeld (Hwa, Oh et al. 1999).

#### *1.5.3.i IGF Binding Protein 2*

The IGFBP-2 gene is located on chromosome 2, encoding a protein of molecular weight approximately 34 -kDa and is highly expressed in fetal CNS tissues. At least in the mouse model, knockout of the IGFBP-2 gene has little phenotypic effect.

#### *1.5.3.ii IGF Binding Protein 4*

The IGFBP-4 gene is located on chromosome 17, and encodes a 237 amino acid protein which is expressed predominantly in liver. The biological role of IGFBP-4 seems predominantly in the regulation of cell proliferation at the growth plate.

#### 1.5.3.iii IGF Binding Protein 5

The IGFBP-5 gene is located on chromosome 5, and encodes a 252 amino acid protein which is expressed predominantly in kidney. IGFBP-5 appears to either enhance or inhibit IGF actions dependent on circumstances and appears particularly important in the regulation of IGF-I action in atretic ovarian follicles and also in bone.

#### 1.5.3.iv IGF Binding Protein 6

The IGFBP-6 gene is located on chromosome 12, and encodes a 216 amino acid protein and binds IGF-II with greater affinity than it does IGF-I. It is present in relatively high levels in cerebrospinal fluids.

#### 1.5.4 *The IGFBP Related Peptides*

The IGFBP<sub>Prps</sub> have distinct structural homology to the IGF binding proteins but bind ligand (IGF-I and IGF-II) at least in vitro, but with much weaker affinity than IGFBPs 1 to 6. To date, up to 9 have been described. They have predominantly isolated from tumour cell lines and have a number of proposed biological functions including the regulation of IGF mediated cellular proliferation and suppression of tumours and vascular disease (Hwa, Oh et al. 1999).

#### 1.5.5 *Quantification of IGF-I*

Conventional laboratory immunoassays for IGF-I quantify total IGF-I, both free IGF-I and IGF-I that is bound to binding proteins, including that incorporated into the ternary complex with ALS. Conventional immunoassays have an initial acidification step during which IGF-I is stripped from the binding proteins before quantification of total (i.e. bound and unbound) IGF-I.

Total IGF-I determination, while useful, gives no information regarding the portion of IGF-I that is unbound and thus readily bio available. The molar ratio of IGF-I to IGFBP-3 has been used as a surrogate for bio available IGF-I, but as it does not take into consideration any of the other binding proteins it is, at best a crude proxy.

Jan Frystyk and Hans Orskov, from Aarhus University in Denmark, have developed a reliable assay for free IGF-I, but it is currently not commercially available (Frystyk, Hussain et al. 1997). Use of this assay provides valuable information regarding bioavailable IGF-I and has been useful in evaluating subtle variation in the growth hormone - IGF-I axis and their relation to observed biological effects (Frystyk, Hussain et al. 1997).

## 1.6. The Growth Hormone - IGF-I Axis in Type 1 Diabetes

### 1.6.1 *Regulation*

#### 1.6.1.i Total IGF-I

Concentrations of circulating IGF-I are low in people with type one diabetes (Edge, Dunger et al. 1990). Circulating IGF-I is predominantly derived from the liver and its release is stimulated by growth hormone (Melmed and Kleinberg 2003). In the non-diabetic, insulin is released from the pancreas directly into the portal circulation and subsequently approximately 70% is cleared as a result of first pass metabolism (Eaton, Friedman et al. 1984). Concentrations of insulin within the portal circulation correlate directly with the expression of the hepatic growth hormone receptor (GHR) and thus in Type 1 Diabetes, where insulin concentrations within the portal circulation are low, the expression of the hepatic GHR is reduced (Shishko, Dreval et al. 1994). In humans this has been confirmed by the low circulating levels of growth hormone binding protein (GHBP); the circulating portion of the hepatic GHR in Type 1 Diabetes (Cheetham, Taylor et al. 1994; Hanaire-Broutin, Sallerin-Caute et al. 1996). The reduced expression of the hepatic GHR leads to a reduction in hepatic IGF-I generation and low circulating levels of IGF-I (Clayton, Holly et al. 1994).

Intraperitoneal administration of insulin in Type 1 Diabetes partially restores the parameters of the growth hormone – IGF-I axis to normal (Hanaire-Broutin, Sallerin-Caute et al. 1996) and when insulin replacement in Type 1 Diabetes is administered directly into the portal circulation, the aberrations of growth hormone and its dependent

peptides are restored to normal (Shishko, Dreval et al. 1994). Currently, portal insulin administration is not a viable therapeutic option in Type 1 Diabetes, however, with the future development of implantable insulin pump devices, future methods of insulin replacement may be much more physiological, not just in terms of dose delivered, but in the anatomical site of delivery.

#### 1.6.1.ii Growth Hormone Hypersecretion

Low concentrations of circulating IGF-I results in a failure of the negative feedback mechanism at the pituitary (Figure 3), resulting in growth hormone hypersecretion that is primarily nocturnal (Edge, Dunger et al. 1990). There is both increased basal growth hormone secretion as well as increased frequency and amplitude of overnight growth hormone pulses when compared to non-diabetic controls matched for age, sex and puberty stage (Edge, Dunger et al. 1990).

#### 1.6.1.iii IGF Binding Proteins and Free IGF-I

In Type 1 Diabetes, concentrations of IGFBP-1 are increased secondary to low concentrations of insulin within the portal circulation and consequent reduced suppression of hepatic generation of IGFBP-1 (Holly, Dunger et al. 1990; Batch, Baxter et al. 1991). IGFBP-1 has a predominantly inhibitory effect on the actions of IGF-I, the increased amounts produced in young people with Type 1 Diabetes are likely to compound the already low total IGF-I concentrations resulting in further reductions in bio available IGF-I (Holly, Smith et al. 1989; Shishko, Dreval et al. 1994).

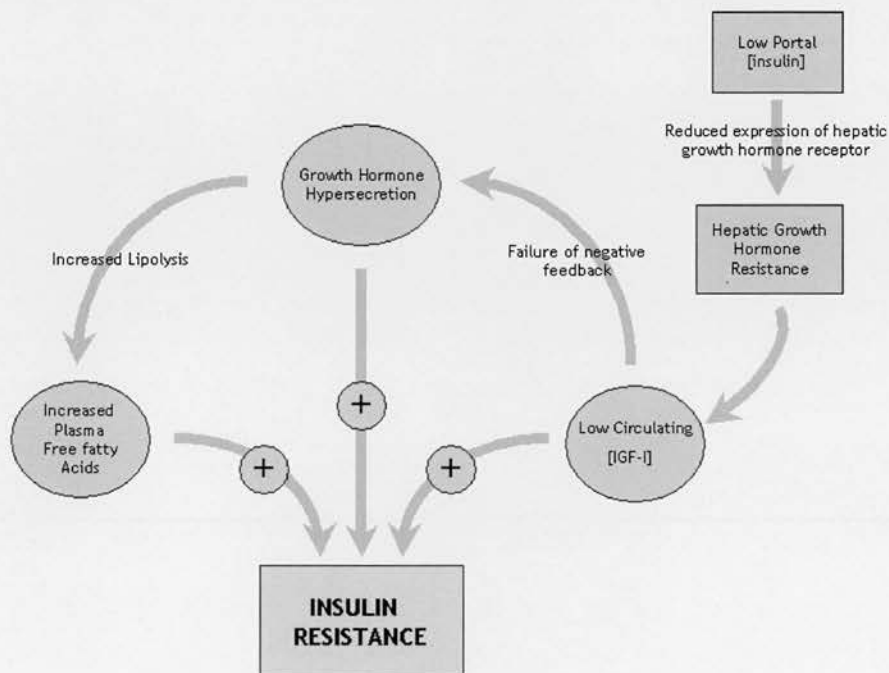
### 1.6.2 *Metabolic Effects*

#### 1.6.2.i Insulin sensitivity

During normal puberty, there is a rise in insulin resistance, which is maximal during mid-puberty and that subsequently falls to pre-pubertal levels during adulthood (Caprio, Cline et al. 1994). This physiological rise in insulin resistance during puberty is accentuated in T1D, with adolescents with T1D being more insulin resistant than control

children matched for age, sex and puberty stage, as determined using hyperinsulinaemic euglycaemic clamp techniques (Amiel, Sherwin et al. 1986).

The exacerbation of the normal pubertal insulin resistance seen in Type 1 Diabetes may be explained by the aberrant regulation of the growth hormone - IGF-I axis described above. Low levels of IGF-I lead to growth hormone hypersecretion, which has a synergistic effect on glucose metabolism; with reduced amounts of bio available IGF-I and increased growth hormone contributing independently to increase insulin resistance.



**Figure 3: The Growth Hormone - IGF-I Axis in Type One Diabetes**

In young people with Type 1 Diabetes; low concentrations of insulin within the portal circulation result in reduced hepatic IGF-I generation with consequent growth hormone hypersecretion. This leads to exacerbates pubertal insulin resistance either by direct or indirect mechanisms.

As discussed earlier in this chapter, growth hormone has direct effects on insulin sensitivity and causes a reduction in insulin sensitivity that is maximal around 5 to 6 hours after a growth hormone pulse (Rizza, Mandarino et al. 1982). In Type 1 Diabetes

this manifests clinically as the 'dawn phenomenon'. Overnight growth hormone secretion is maximal in the early hours of the night (at around 0100h) and this results in increased insulin resistance, with an increase in insulin requirements during the late part of the night with resultant fasting hyperglycaemia (Edge, Dunger et al. 1990). Practically speaking, in terms of management this can lead to problems as conventional insulin preparations have maximal effect in the early hours of the night, when insulin requirements are at their lowest, but their actions are waning as insulin requirements rise during the latter part of the night and thus increasing the evening dose of insulin in an attempt to control morning hyperglycaemia may result in unacceptable hypoglycaemia during the early part of the night and this may be compounded if soluble insulin is used in conjunction with Neutral Protamine Hagedorm (NPH) insulin. The problem with nocturnal hypoglycaemia may be partially resolved if soluble insulin is replaced with a rapid acting analogue such as insulin *lispro* (Ford-Adams, Murphy et al. 2003).

#### 1.6.2.ii Lipolysis

As well as having direct effects on insulin sensitivity, growth hormone has indirect effects on insulin sensitivity that are mediated via its effects on lipolysis. Growth hormone acts to stimulate lipolysis with subsequent release of free fatty acids (Arslanian and Kalhan 1994; Piatti, Monti et al. 1999). Increased concentrations of circulating free fatty acids contribute to reductions in insulin sensitivity via the competitive inhibition of GLUT 4 mediated glucose uptake at cell membranes (Shulman 2000). Increased rates of lipolysis overnight have been demonstrated in young people with Type 1 Diabetes, and while this may be secondary to suboptimal insulinisation overnight, growth hormone hypersecretion is likely to be a contributory factor (Hagstrom-Toft, Bolinder et al. 1997).

#### 1.6.3 Summary

In summary, the growth hormone - IGF-I axis is aberrantly regulated in young people with Type 1 Diabetes. The peripheral administration of insulin leads to reduced hepatic



IGF-I generation and thus to reduced circulating concentrations of IGF-I which in turn leads to growth hormone hypersecretion due to reduced negative feedback at the pituitary. The low concentrations of IGF-I are compounded by increased levels of IGFBP-1 (the main inhibitory binding protein for IGF-I) resulting in a further reduction in IGF-I bio availability. Low concentrations of IGF-I and growth hormone hypersecretion may compound difficulties with management, by causing insulin resistance, both directly and indirectly, by the stimulation of lipolysis. Administration of insulin directly into the portal circulation has been shown to restore the parameters of the growth hormone - IGF-I axis to normal in Type 1 Diabetes; however this is not currently a reasonable therapeutic option.

### 1.7. Nephropathy in Diabetes

#### 1.7.1 *Incidence and Implications*

Diabetes (types one and two) is the most common cause of end stage renal failure in the developed world and accounts for approximately half of all cases, with a considerable associated economic burden [[http://www.usrds.org/adr\\_2004.htm](http://www.usrds.org/adr_2004.htm)]. (US Renal Data System, 2004). The cumulative incidence of nephropathy increases progressively from diagnosis but plateaus at around 40%, which may indicate that certain individuals are vulnerable to the development of nephropathy while others are protected, no matter how bad their diabetes control (Hovind, Tarnow et al. 2004). Investigation of current candidate genetic polymorphisms is underway in an attempt to identify those potentially at risk of nephropathy and target them for early and aggressive intervention (Chowdhury, Dronsfield et al. 1996; Chowdhury, Dyer et al. 1998).

#### 1.7.2 *Natural history*

The herald sign of renal disease in type one diabetes is glomerular hyperfiltration; first noted by Cambier in 1934 when he reported that subjects with newly diagnosed Type 1 Diabetes had greater inulin clearances than controls (Cambier 1934). Glomerular hyperfiltration has subsequently been confirmed by numerous authors (Ditzel and

Schwartz 1967; Mogensen 1971). The period of hyperfiltration is transient, and is associated with increased renal size on ultrasound (Christiansen, Gammelgaard et al. 1981; Mogensen, Christensen et al. 1990). It is unclear what mechanism drives the hyperfiltration and numerous candidates have been proposed, including hyperglycaemia. Hyperfiltration appears to affect only 30 to 40% of young people with Type 1 Diabetes and intensification of insulin treatment with reduction in hyperglycaemia leads to a decline in GFR over successive weeks (Christiansen, Giese et al. 1988). It is possible that glomerular hyperfiltration may be an early manifestation of an individual's predisposition to hyperglycaemic renal insult.

Longitudinal studies have demonstrated that early hyperfiltration predicts risk of subsequent nephropathy (Rudberg, Persson et al. 1992). Recent work from the Oxford cohort of young people with type one diabetes (ORPS) confirms this, with those who subsequently go on to develop microalbuminuria having significantly higher GFR five years from diagnosis than those who do not develop MA, independently of HbA1c and duration of diabetes (Amin, Turner et al. 2005).

Around 18 to 24 months from diagnosis, glomerular lesions can be seen on biopsy, but urinary albumin excretion rate (AER) remains within the normal range ( $< 25\text{mg} / 24\text{hrs}$ ). Lesions are characterised by thickening of the glomerular basement membrane, coupled with expansion of the mesangial matrix (Osterby, Bangstad et al. 1996).

The first clinical manifestation of diabetic nephropathy is microalbuminuria (MA), a persistent elevation in AER which is incipient and in the range 30 to 300 mg / 24 hr which subsequently climbs into the frankly nephrotic range ( $\text{AER} > 300\text{mg}/24\text{hrs}$ ). Urinary albumin excretion varies greatly from day to day within individuals, being increased following exercise and brief periods of very poor metabolic control (Cohen, Close et al. 1987). Because of this, MA should not be diagnosed unless it has been present in 2 out of 3 timed urine collections on at least 2 separate occasions during a 6 month period (Schultz, Amin et al. 2002). Microalbuminuria is rare before puberty, but

thereafter its incidence increases resolutely and is often associated with abnormalities in control of blood pressure; initially with loss of normal nocturnal dipping in systolic blood pressure (only apparent with 24 hour ambulatory blood pressure monitoring), with subsequent frank hypertension (Darcan, Goksen et al. 2006). In addition to intensification of insulin therapy and optimisation of glycaemic control, therapy with an angiotensin converting enzyme (ACE) inhibitor confers renal protection at this stage (Laffel, McGill et al. 1995).

An albumin excretion rate of 300mg / 24hrs, defines frank nephropathy and is accompanied by a progressive decline in GFR exacerbated by hypertension (Rudberg and Osterby 1997). Subsequent end stage renal failure with need for dialysis and renal transplantation is almost inevitable in those with nephropathy after 20 to 30 years diabetes duration.

### *1.7.3 Risk factors*

The main risk factor for the development of diabetic kidney disease is poor control; risk of nephropathy has been shown to correlate directly with HbA1c in many studies (Rudberg and Dahlquist 1996). Intervention has likewise been shown to reduce nephropathy risk; the landmark Diabetes Control and Complications Trial (DCCT) demonstrated that aggressive diabetes management with intensive insulin therapy to improve glycaemic control and reduce HbA1c resulted in significantly lower risk of nephropathy development in a cohort of adolescents with type one diabetes (1994).

Although the importance of glycaemic control as a primary determinant of complications risk cannot be ignored, there are other factors that appear to be important in determining risk of nephropathy. Diabetes duration, angiotensin converting enzyme genotype, hypertension, smoking, family history of hypertension and female sex may all confer risk of nephropathy, independently of HbA1c (Rudberg and Dahlquist 1996). The Oxford Regional Prospective Study (ORPS) has followed a large cohort of young people with type one diabetes followed longitudinally from diagnosis, with annual

determination of HbA1c, albumin creatinine ration (ACR), sex hormone binding globulin and parameters of the GH/IGF-I axis (Schultz, Konopelska-Bahu et al. 1999). Cases with microalbuminuria (MA) were then compared with MA negative controls matched for age, sex, and duration of diabetes. Independently of HbA1c, female sex and puberty stage were independently associated with MA (Schultz, Konopelska-Bahu et al. 1999). Additionally, in the females, MA risk was associated with a lower sex hormone binding globulin (ie increased free androgen index) (Amin, Schultz et al. 2003). Further analysis within the same cohort revealed that subjects with MA had a significantly lower free IGF-I concentration than MA negative controls matched for age, sex, puberty stage and duration of diabetes, independently of HbA1c (Amin, Schultz et al. 2003).

#### *1.7.4 The role of Growth Hormone and IGF-I in Diabetic Nephropathy*

Historically it was noted that patients with both diabetes and growth hormone deficiency were protected against the microvascular complications of diabetes; retinopathy and nephropathy. Indeed, hypophysectomy was at one time used in the treatment of severe retinopathy, a treatment that has now (thankfully!) been obviated by the development of laser ablation of neovascularisation. In addition, patients with acromegaly exhibit glomerular hyperfiltration and increased urinary albumin excretion, particularly during exercise (Hoogenberg, Sluiter et al. 1993; Manelli, Bossoni et al. 2000). Subjects with Type 1 Diabetes and glomerular hyperfiltration ( $GFR > 130 \text{ ml/min/1.73m}^2$ ) have higher overnight growth hormone secretion than those with normofiltration (Blankestijn, Derkx et al. 1993). More recently, young adults with MA from a large longitudinal cohort study have been shown to have increased overnight growth hormone secretion than those without MA, in addition to lower concentrations of IGF-I (Amin, Williams et al. 2005).

In Type 1 Diabetes, hyperfiltration precedes the development of MA and the subsequent decline in GFR and frank nephropathy. Hyperfiltration soon after the diagnosis of Type 1 Diabetes may also predict MA (Amin, Turner et al. 2005; Steinke, Sinaiko et al. 2005).

Thus growth hormone hypersecretion could have a role in the pathogenesis of early diabetic nephropathy. Abnormalities of the growth hormone – IGF-I axis similar to those seen in humans are seen in a mouse model of diabetes in which treatment with a specific growth hormone antagonist leads to reductions in GFR and urinary albumin excretion, secondary to a reduction in renal expression of IGF-I (Flyvbjerg, Bennett et al. 1999).

Although there is hepatic resistance to growth hormone, the integrity of GHR expression in peripheral tissues such as the kidney remains intact (Flyvbjerg 2000). In the face of exaggerated growth hormone secretion, increased paracrine production of IGF-I in peripheral tissues occurs (Segev, Landau et al. 1997). Studies in humans have suggested that subjects with MA have higher urinary IGF-I than controls without MA, despite the fact that circulating serum levels of free IGF-I are lower in patients with MA than their matched controls without MA (Cummings, Sochett et al. 1998; Amin, Ong et al. 2001). However, urinary data need to be interpreted with caution as urinary concentrations of peptide hormones depend not only on local concentrations within the kidney but also on tubular function and have to be viewed with a degree of scepticism in the light of a kidney which is leaking albumin, a much larger protein (Hourd, Edge et al. 1991). Despite this, there is mounting evidence from rodent models of diabetes that nephropathy correlates directly with IGF-I expression within the kidney (Flyvbjerg 2000).

#### 1.8. The Growth Hormone - IGF-I Axis in Type 1 Diabetes: Potential Strategies for Manipulation

The abnormalities of the growth hormone - IGF-I axis in type one diabetes may lead to increased insulin resistance, increased lipolysis and contribute to the development of the complications of diabetes around puberty. Manipulation of the growth hormone - IGF-I axis in an attempt to restore more normative physiology may provide an additional



strategy by which to improve insulin sensitivity and reduce the risk of the microvascular complications. There are a number of potential ways of approaching this.

### *1.8.1 Specific Growth Hormone Receptor Blockade*

#### *1.8.1.i Specific Growth Hormone antagonists*

Recombinant DNA technology has facilitated generation of powerful growth hormone analogues which bind to the growth hormone receptor with high affinity, but do not activate downstream signalling; growth hormone antagonists (GHA). B2036-PEG (Pegvisomant, Somavert, Pharmacia) is a GHA developed for use in refractory acromegaly and incorporates mutations of 9 amino acids in the native human growth hormone molecule. The addition of a polyethylene glycol moiety (pegylation) increases the circulating half-life of the molecule to over 2 days and also reduces its immunogenicity. B2036-PEG binds to the GHR with greater affinity than does the native human growth hormone molecule, but does not induce the functional conformational change within the receptor, which is required for downstream signalling (Figure 4) (Thorner, Strasburger et al. 1999).

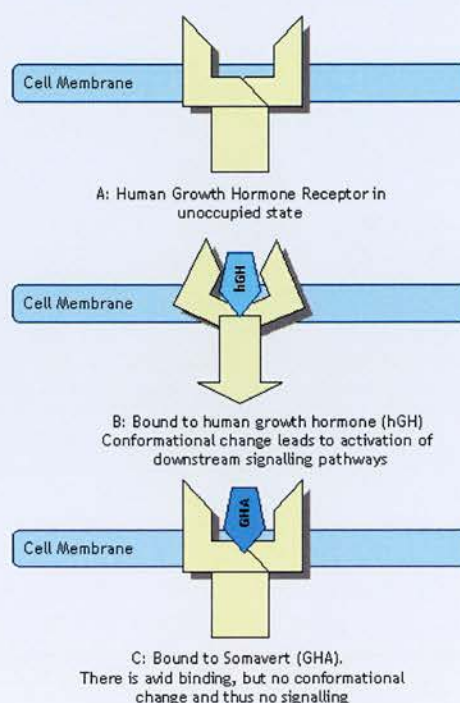
Despite binding to the receptor with greater affinity, much higher concentrations of B2036-PEG are required for reductions in IGF-I both in healthy control subjects and in subjects with acromegaly (Thorner, Strasburger et al. 1999; Trainer, Drake et al. 2000). Detailed studies of receptor binding indicate that modification of the molecule by Pegylation in order to reduce immunogenicity and prolong half life may lead to a reduced affinity for the receptor dimer (Ross, Leung et al. 2001).

#### *1.8.1.ii B2036-PEG: preliminary data*

Phase 1 studies of B2036-PEG administration to healthy males demonstrated that a single dose of 1mg/kg was well tolerated, and led to reductions in IGF-I concentrations by approximately 50% after 5 days (Thorner, Strasburger et al. 1999). In this study, the authors reported that there was no increase in endogenous growth hormone secretion



following B2036-PEG administration, and were careful to use assays for which there was no cross reactivity between endogenous growth hormone and B2036-PEG by (Thorner, Strasburger et al. 1999). It is likely that if conventional ELISA kits incorporating polyclonal antibodies are used, that there would be cross reactivity between endogenous growth hormone and the B2036-PEG molecule and the manufacturers (Pfizer) recommend that in acromegaly, IGF-I concentrations, rather than quantification of growth hormone is used for monitoring and dose titration.



**Figure 4: The Human Growth Hormone Receptor**

Effects of binding of natural ligand (hGH) and specific growth hormone antagonist (GHA).

#### 1.8.1.iii B2036-PEG: studies in acromegaly

Studies in patients with acromegaly have shown the GHA B2036-PEG, Somavert (Pharmacia Corporation) to effectively block the growth hormone receptor and reduce concentrations of circulating IGF-I (dose 5 to 20 mg) (Trainer, Drake et al. 2000). The drug is well tolerated and has few side effects (Trainer, Drake et al. 2000) and may enhance insulin sensitivity, in contrast to the somatostatin analogue, octreotide, which

has unpleasant side effects and may exacerbate insulin resistance (Rose and Clemmons 2002; Drake, Rowles et al. 2003). Acromegaly is characterised by reductions in total cholesterol and LDL concentrations which are reversed by treatment with B2036-PEG in conjunction with reductions in fasting insulin concentrations (Parkinson, Drake et al. 2002).

#### 1.8.1.iv Growth hormone blockade in animal models of diabetes

An experimental model of Type 1 Diabetes in mice has abnormalities of the growth hormone – IGF-I axis similar to those seen in humans (Landau, Domene et al. 1998). Administration of a GHA to mice with nephropathy leads to a reduction in renal expression of IGF-I, renal size, GFR, ERPF and urinary albumin excretion rates (Flyvbjerg, Bennett et al. 1999; van Neck, Dits et al. 2000). Thus far, no studies of the effect of GHAs on insulin sensitivity, renal physiology or tubular protein leak have been performed in humans.

Selective blockade of the actions of growth hormone in young people with type one diabetes may lead to improvements in insulin sensitivity and subsequently to reductions in insulin requirements. In addition, blockade of the growth hormone receptor in the kidney may afford renal protection and provide an alternative therapeutic strategy in this vulnerable population.

#### 1.8.2 *IGF-I Administration*

##### 1.8.2.i rhIGF-I therapy in humans with Type 1 Diabetes

rhIGF-I has been explored as an adjunctive therapy and the results of previously published studies are summarised in Table 1 (Thraillkill KM FAU - Quattrin, Quattrin T FAU - Baker et al.; Cheetham, Holly et al. 1995; Acerini, Patton et al. 1997; Carroll, Umpleby et al. 1997; Cheetham, Connors et al. 1997; Quattrin, Thraillkill et al. 1997; Acerini, Harris et al. 1998; Crowne, Samra et al. 1998; Quattrin, Thraillkill et al. 2001; Simpson, Jackson et al. 2004). Administration of recombinant human IGF-I (rhIGF-I) to

young adults with Type 1 Diabetes results in suppression of overnight growth hormone secretion (Acerini, Harris et al. 1998). Detailed physiological studies of the administration of exogenous rhIGF-I (40mcg/kg/day) to adolescents with Type 1 Diabetes demonstrated reductions in overnight insulin requirements for euglycaemia, in conjunction with reductions in overnight plasma insulin concentrations and increased IGFBP-1 concentrations (Cheetham, Jones et al. 1993; Cheetham, Clayton et al. 1994). Hyperinsulinaemic euglycaemic clamp studies using cold isotopes to model glucose turnover revealed an increase in insulin sensitivity during the clamp following IGF-I administration, which appeared to be predominantly secondary to an effect on hepatic glucose production (Acerini, Harris et al. 1998).

Longer-term clinical trials of adjunctive therapy with rhIGF-I were also carried out in young people with Type 1 Diabetes. A randomised, double-blind placebo-controlled trial of IGF-I at a dose of 40mcg/kg/day for 6 months reported a reduction in HbA1c after 3 months but no change in overall daily insulin requirement and the improvement in HbA1c did not persist until the end of the trial which may reflect concordance issues (Acerini, Patton et al. 1997). Importantly, at this physiological replacement dose of IGF-I, there were no serious adverse events reported and the risk of hypoglycaemia was not greater in the IGF-I treated group over the control group.

Table 1. Effects of adjunctive therapy with rhIGF-I in subjects with Type 1 Diabetes Mellitus				
Authors	N	IGF-I dose (mcg/kg/24hrs)	Duration of therapy	Principle Outcomes
(Cheetham, Holly et al. 1995)	6	40	28 days	Increased IGF-I concentrations. Reductions in HbA1c and insulin requirements
(Acerini, Patton et al. 1997)	53	20-40	24 weeks	Increased IGF-I concentrations. Reductions in HbA1c but no effect on insulin requirements
(Cheetham, Connors et al. 1997)	16	40	24 hours	Increased IGF-I concentrations. Reductions in overnight growth hormone secretion and insulin concentrations
(Quattrin, Thrailkill et al. 1997)	43	80	4 weeks	Increased IGF-I concentrations. Reductions in HbA1c, glucose concentrations and insulin dose
(Acerini, Harris et al. 1998)	7	20-40	24 hours	Increased IGF-I concentrations. Reductions in overnight growth hormone secretion and insulin concentrations. Increased hepatic insulin sensitivity.
(Crowne, Samra et al. 1998)	9	40	24 hours	Increased IGF-I concentrations. [Endogenous GH secretion was suppressed using octreoType 1 Diabetes and GH was given back as pulses throughout the night]. Reduced overnight insulin requirements.
(Carroll, Umpleby et al. 1997)	6	100	19 days	Increased IGF-I concentrations, reduced GH secretion, decreased insulin concentrations, total cholesterol and triglycerides
(Thrailkill, Quattrin et al. 1999)	22 3	80-140	12 weeks	Administration of rhIGF-I compared with intensification of standard insulin therapy. rhIGF-I group had lower HbA1c with similar rates of hypoglycaemia.
(Quattrin, Thrailkill et al. 2001)	55	80-140	12 weeks	Reduced HbA1c and reduced insulin requirements. Dose related worsening of retinopathy.
(Simpson, Jackson et al. 2004)	11	40	24 hours	Increased hepatic and peripheral insulin sensitivity but no effect on lipid metabolism.

#### 1.8.2.ii rhIGF-I therapy in humans with T2DM

Adjunctive therapy with rhIGF-I has been studied in subjects with T2D by a number of groups and details are summarised in Table 2 (Zenobi, Jaeggi-Groisman et al. 1992; Schalch, Turman et al. 1993; Jabri, Schalch et al. 1994; Moses, Young et al. 1996; Cusi and DeFronzo 2000). Doses administered ranged between 160 and 300 mcg/kg/day (much greater than the doses used in the Type 1 Diabetes population) and exposure was generally restricted to short-term (5 to 7 days) with physiological evaluation of insulin sensitivity before and after exposure, but two groups have reported exposure for 6 weeks. With only one exception (Young and Clemmons 1994), authors report improvements in insulin sensitivity, fasting glucose and response to oral glucose load and more detailed physiological studies suggest an effect both on hepatic and peripheral insulin sensitivity (Dunger, Yuen et al. 2004).

#### 1.8.2.iii Safety issues

The development of unbound rhIGF-I as a potential therapeutic option in Type 1 Diabetes was discontinued due to concerns about the role of IGF-I in the microvascular complications of Type 1 Diabetes and also due to dose-related side effects. Large IGF-I doses (above 80 µg/kg/day) have been associated with a dose-dependent increase in side effects, including oedema, jaw pain, arthralgia, and early worsening of retinopathy (Quattrin, Thrailkill et al. 2001). Concern regarding the association of IGF-I, particularly with retinopathy resulted in it being withdrawn from development as a potential therapy in either type 1 or type 2 diabetes.

More recently, rhIGF-I has been complexed to rhIGFBP-3, its natural carrier protein (Mecasermin rinfabate, IPLEX™) in an attempt to prolong half-life and reduce the risk of short-term peaks of free IGF-I. The combination of rhIGF-I with its natural binding protein, rhIGFBP-3 may allow the efficacy of rhIGF-I to be realised without the associated toxicity.



<i>Table 2. Effects of adjunctive therapy with rhIGF-I in subjects with Type 2 Diabetes Mellitus</i>				
Authors	N	IGF-I dose (mcg/kg/24hrs)	Duration of therapy	Principle Outcomes
(Zenobi, Graf et al. 1992)	6	240	5 days	Increased fasting insulin sensitivity and reductions in area under curve for glucose, insulin and C-peptide during oral glucose challenge.
(Schalch, Turman et al. 1993)	12	180-320	5 days	Increased fasting insulin sensitivity and reductions in area under curve for glucose, insulin and C-peptide during oral glucose challenge. Reductions in triglycerides and total cholesterol.
(Jabri, Schalch et al. 1994)	7	240-320	4-52 days	Trial discontinued due to concern regarding incidence of side effects such as oedema of the hands. Improved glycaemic control in
(Young and Clemmons 1994)	6	160-300	4 days	Increased IGFBP-1, reductions in IGFBP-3, with reduced area under the curve C-peptide. No effect on fasting insulin sensitivity.
(Moses, Young et al. 1996)	12	200	6 weeks	Decreased HbA1c and increased fasting insulin sensitivity. Increased insulin sensitivity as determined by intravenous glucose tolerance test.
(Cusi and DeFronzo 2000)	8	160	7 days	Increased fasting insulin sensitivity. Increased hepatic and peripheral insulin sensitivity as determined by hyperinsulinaemic clamp studies.

#### 1.8.2.iv rhIGF-I/rhIGFBP-3 complex: Mecasermin Rinfabate

rhIGF-I/rhIGFBP-3 complex (product name IPLEX<sup>TM</sup>, generic name Mecasermin rinfabate, previously marketed as Somatokine) is a recombinant protein complex of IGF-I and IGFBP-3 combined in equimolar proportions. It currently does not carry a license for any therapeutic intervention. In animal studies, Mecasermin rinfabate displays metabolic and anabolic actions similar to those observed when rhIGF-I is administered alone (Adams, Moore et al. 1995; Bagi, van der Meulen et al. 1995). The ability of



Mecasermin rinfabate to form a ternary complex with ALS (acid labile subunit) provides a mechanism to maintain rhIGF-I in a bound form in the circulation, thereby prolonging its half-life and preventing the undesirable acute pharmacological effects that have been observed following the administration of rhIGF-I alone (Adams, Moore et al. 1995).

Mecasermin rinfabate is produced by two strains of *E. coli*, one producing IGF-I and the other, IGFBP-3. The two proteins then undergo a purification process and are combined into a complex in equimolar proportions. At near neutral pH, the dissociation constant is approximately 50 pM. Mecasermin rinfabate (marketed as IPLEX™) was developed by Inmed Incorporated (PO box 2400, Glen Allen, VA 23058, USA) in the hope that it would provide a more stable pool of circulating IGF-I within the circulation, potentially improving the pharmacokinetic profile, and reducing the incidence of significant side effects (Norman 2003).

Mecasermin rinfabate has been administered by subcutaneous injection once daily in doses of between 0.1mg/kg/day up to 2 mg/kg/day, depending on the indication [1mg Mecasermin rinfabate equates to approximately 0.2mg of rhIGF-I]. Once dissociated from IGFBP-3, the rhIGF-I derived from Mecasermin rinfabate should bind to receptors in an identical fashion to endogenous IGF-I, with indistinguishable biological effects.

In vitro animal studies demonstrated that the rhIGF-I/rhIGFBP-3 complex mimics the physiological effects of IGF-I with a longer half-life (Sanders, Moore et al. 1997). In animal studies the the rhIGF-I/rhIGFBP-3 complex has biological effects similar to or enhanced when compared to IGF-I alone (Bagi, Brommage et al. 1994; Adams, Moore et al. 1995; Bagi, van der Meulen et al. 1995; Bagi, DeLeon et al. 1995). On entering the circulation the the rhIGF-I/rhIGFBP-3 complex binds to ALS to form the ternary complex that forms the natural reservoir for circulating IGF-I. These preliminary experimental studies were followed by Phase 1 and 2 clinical trials, which demonstrated its safety and efficacy in adult healthy volunteers and subjects with type 1 and 2 diabetes mellitus, patients recovering from osteoporotic hip fracture and children with Growth

Hormone Insensitivity Syndrome (GHIS) (Clemmons, Moses et al. 2000; Boonen, Rosen et al. 2002; Saukkonen, Amin et al. 2004; Clemmons, Moses et al. 2005; Saukkonen, Shojaee-Moradie et al. 2006; Williams, Yuen et al. 2006; Clemmons, Sleevi et al. 2007).

The dose of Mecasermin rinfabate has been titrated according to the individual subject, but in most cases, the manufacturers recommend a starting dose of 0.5mg/kg/day, to be titrated upwards in 0.5mg/kg/day increments, to a recommended maximum of 2mg/kg/day. In GHIS, the dose can be titrated to maintain IGF-I concentrations of the order of +2 standard deviation scores for age, based on plasma IGF-I concentration taken approximately 18 hours following a dose (Camacho-Hubner, Underwood et al. 2006). Injections should be given at approximately the same time every day, but can be given either morning or evening, whichever is more convenient for the individual.

Once within the circulation, the rhIGF-I from the complex can form a ternary complex with IGFBP-3 and Acid labile subunit (ALS) and is subsequently cleared from the circulation by the same means as native IGF-I. Most studies reporting the administration of Mecasermin rinfabate to humans report dose dependent increases in IGF-I, but with less marked rises in IGFBP-3 (Clemmons, Moses et al. 2000; Clemmons, Moses et al. 2005; Saukkonen, Shojaee-Moradie et al. 2006; Clemmons, Sleevi et al. 2007). This has led to speculation concerning possible proteolytic degradation of the non-glycosylated form of the recombinant IGFBP-3. Endogenous IGFBP-3 is subject to degradation by protease activity which provides a further level of regulation both of IGFBP-3 concentrations and functional capacity, and hence IGF-I bioavailability (Baxter, Suikkari et al. 1993). IGFBP-3 protease activity was initially described in the serum of pregnant women; proteolysis of IGFBP-3 leads to the formation of fragments that bind IGF-I with reduced affinity, thus increasing the bioavailability of IGF-I (Baxter, Suikkari et al. 1993). IGFBP-3 proteases have not yet been fully characterised but are thought to be serine, or metallo-proteases (Bunn and Fowlkes 2003; Fowlkes, Serra et al. 2004). Thus

regulation of IGFBP-3 synthesis and of its subsequent proteolysis under certain conditions permits a dynamic regulation of bio available IGF-I but the exact nature of its significance or the mechanisms which induce proteolysis remain uncertain.

Work by Clemmons *et al* using immunoblotting techniques (Clemmons, Sleevi et al. 2005) suggests that, following administration Mecasermin rinfabate (containing the recombinant form of IGFBP-3), the non-glycosylated protein is preferentially degraded by plasma proteases, preferentially leaving the native glycosylated form of the protein.

### 1.8.3 *Suppression of growth hormone secretion*

#### 1.8.3.i Somatostatin and its analogues

Suppression of endogenous growth hormone secretion using the long acting somatostatin analogue octreotide has been reported to reduce insulin requirements by up to 50% in several clinical trials (Navascues, Gil et al. 1988; Kirkegaard, Norgaard et al. 1990; Orskov, Moller et al. 1996). However, benefits were limited by the development of unacceptable side effects such as hypoglycaemia and gastrointestinal upset, which occur as a result of somatostatin's ubiquitous effects on hormone secretion.

#### 1.8.3.ii Pirenzepine

Short-term clinical studies using the anti-cholinergic agent pirenzepine have also shown effective suppression of growth hormone secretion with improvements in insulin sensitivity (Martina, Maccario et al. 1989; Atiea, Aslan et al. 1990; Aman, Kroon et al. 1996). Improvements in insulin sensitivity appeared to be mediated via an effect on lipolysis (Halldin, Brismar et al. 2002). However, the short half-life of pirenzepine limited its efficacy and attempts to prolong the half-life resulted in an exacerbation of side effects, making it an unsuitable agent for long-term therapy (Krassowski, Szulc et al. 1993).

#### *1.8.4 Inhibition of Lipolysis*

Reduction of circulating NEFA concentrations by using lipolysis inhibitors such as the agent acipimox have been shown to have favourable effects on insulin sensitivity in healthy controls and in subjects both with impaired glucose tolerance and type 2 diabetes (Fulcher, Catalano et al. 1992; Fulcher, Walker et al. 1993; Walker, Agius et al. 1993). Acipimox therapy has also been shown to reverse the decline in insulin sensitivity seen with exogenous growth hormone therapy in individuals with growth hormone deficiency (Nielsen, Moller et al. 2001). However, to date there are no studies of adjunctive therapy with acipimox in Type 1 Diabetes.

#### 1.9. Aims of this Thesis

The work reported in this thesis aims to gain further insight and knowledge into the growth hormone - IGF-I axis in young adults with Type 1 Diabetes. Detailed physiological studies before and after specific growth hormone blockade will extend knowledge and understanding of the physiological impact of growth hormone hypersecretion on glucose and lipid metabolism in young people with Type 1 Diabetes and may ultimately facilitate development of a viable adjunctive therapy which may improve glycaemic control and afford protection against the risk of the complications of diabetes.

Previous studies have demonstrated that administration of exogenous IGF-I at physiological doses to young people with Type 1 Diabetes leads to suppression of growth hormone secretion and enhancement of insulin sensitivity. However, concern regarding the safety profile of IGF-I when administered at high doses led to its withdrawal from development as a potential therapy in Type 1 Diabetes. More recently, rhIGF-I complexed to rhIGFBP-3 has been developed as a means of prolonging the half-life of IGF-I and reducing the risk of potentially dangerous side effects. The work reported in this thesis should increase understanding of the pharmacokinetics of rhIGF-

I/rhIGFBP-3 complex, particularly effects on plasma concentrations of IGFBP-3 and on IGFBP-3 proteolysis.

Finally, growth hormone hypersecretion has been implicated in the pathogenesis of the complications of diabetes, and may lead to glomerular hyperfiltration and microalbuminuria. Detailed physiological evaluation of renal haemodynamics and urinary albumin excretion rates following suppression of endogenous growth hormone secretion by the exogenous administration of IGF-I complexed to its natural carrier protein; IGFBP-3 will increase understanding of growth hormone's role in the glomerular hyperfiltration and renal disease in type one diabetes.

## **Chapter 2: SPECIFIC GROWTH HORMONE RECEPTOR BLOCKADE IN Type 1 Diabetes**

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### **2.1. Introduction**

This chapter will describe a physiological study undertaken using the specific growth hormone antagonist; B2036-PEG (Pharmaceutical name Pegvisomant, Brand name Somavert®, Pfizer, UK) in young adults with type one diabetes. The study was of randomised crossover design, comparing the effects of 2 doses of B2036-PEG (5 and 10mg) on insulin sensitivity and glucose and lipid metabolism, assessed at baseline and following each of 2 treatment blocks.

### **2.2. Rationale**

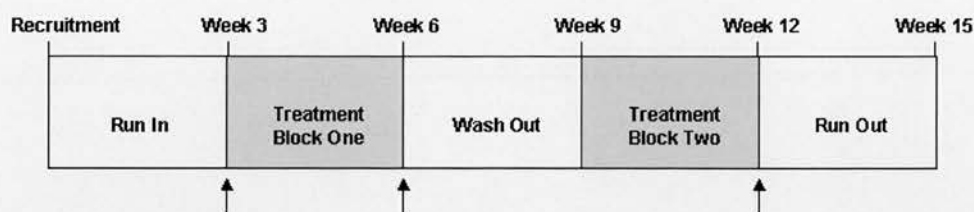
The preliminary data from animal and human studies suggest that B2036-PEG can effectively block the actions of growth hormone, and is relatively free of side effects. By blocking the adverse metabolic effects of growth hormone on carbohydrate and lipid metabolism, despite reducing IGF-I concentrations, adjunctive therapy with B2036-PEG may improve insulin sensitivity in Type 1 Diabetes.

### **2.3. Methods**

#### **2.3.1 *Study Design***

The study was an investigator blinded, randomised crossover study of two 3-week treatment periods with either 5 or 10mg B2036-PEG administered once daily via subcutaneous injection for 3 weeks, separated by a 3-week washout period. There was a 3-week run in period at the beginning and a 3-week run out at the end. The study schedule is shown in Figure 5.





**Figure 5: Study Schedule**

Schematic representation of the study schedule. Arrows denote the timing of overnight euglycaemic clamp studies. Each subject received treatment with both doses (5 and 10mg) of B2036-PEG in random order.

### 2.3.2 Subjects

8 young adult subjects with Type 1 Diabetes were recruited for the study from diabetes clinics in Cambridge, Aylesbury and Northampton, (3M, median age 18.7 years; (range, 17.3 to 22.6); median body mass index 26.7 Kg/m<sup>2</sup>; (range 22.2 to 28.2); median HbA1c 9.4% (range, 8.0-10.5)). All were post pubertal, with Type 1 Diabetes of at least 2 years' duration, and were C-peptide negative. All had normal renal, hepatic and thyroid function and a normal full blood count. None had any evidence of microalbuminuria or retinopathy. All were treated with a combination of short and long acting insulins two to four times per day (median insulin dose 0.9u/Kg/24hrs (range 0.5-1.1)). Details of individual subjects are given in Table 3.

The study protocol was approved by the Cambridge, Aylesbury and Northampton Local Research Ethics Committees. Written, informed consent was obtained from all subjects prior to the study. All overnight studies were performed at Addenbrooke's Hospital, Cambridge, UK in the Wellcome Trust Clinical Research Facility.

<i>Table 3. Subject Characteristics</i>							
Subject ID	Treatment Order #	Insulin Dose (u/kg/day)	Regimen	Age (yr)	BMI (kg/m <sup>2</sup> )	Sex	HbA1c (%)
1	1	0.48	bd	18.5	27.1	F	10.5
2 <sup>α</sup>	2	1.08	bd	17.5	23.2	F	10.0
3	1	1.14	MIT	18.0	28.2	M	8.6
4	2	0.52	MIT	17.7	22.2	F	7.2
5	2	0.90	MIT	18.1	26.7	F	10.1
6	1	0.51	MIT	22.6	24.8	M	8.0
7	2	0.99	MIT	17.3	27.3	M	10.8
8	1	0.94	MIT	18.9	24.0	F	9.4
# 1: Treatment order 5mg/day B2036-PEG in first block and 10mg/day B2036-PEG in second block 2 Treatment order 10mg/day Somavert in first block and 5mg/day Somavert in second block  α Subject 2 was excluded from analysis due to failure to omit long acting insulin prior to one of the study nights, thus invalidating any data.							

### 2.3.3 Study Timetable

#### 2.3.3.i Run in Period

Baseline HbA1c, toxicology (full blood count, liver function tests and urea and electrolytes), IGF-I and IGFBP-3 were measured at screening. During the run in period, subjects were contacted weekly by phone and insulin dose was titrated according to home blood glucose monitoring to achieve optimal glycaemic control. A baseline overnight assessment of insulin sensitivity and glucose and lipid metabolism, followed by hyperinsulinaemic clamp was performed at the end of this period (please see section 2.3.4 for details).

#### 2.3.3.ii Treatment periods

During each treatment period, subjects were randomised to receive either 5 or 10mg of B2036-PEG (S5 and S10 respectively) once daily via subcutaneous injection in addition to their usual insulin regimen. The investigator was blinded as to the order in which each subject received the doses until after the completion of the study. At the beginning of each treatment block, subjects were advised to reduce their total daily insulin dose by

20% in order to minimise the risk of hypoglycaemia. Thereafter, insulin requirements were titrated according to home blood glucose monitoring following telephone liaison with the investigator (please see Appendix 1 for details). Overnight assessments of insulin sensitivity and glucose and lipid metabolism, followed by hyperinsulinaemic clamp, identical to that performed at baseline, were performed on day 21 of each treatment period (please see section 2.3.4 for details).

#### 2.3.3.iii Wash out and Run Out Periods

During these periods, subjects received their usual insulin regimen and regular telephone contact was maintained with the investigator with insulin dose being titrated according to home blood glucose monitoring.

#### 2.3.4 *Overnight Variable Rate Insulin Infusion*

For the 36 hours prior to admission for each overnight study, all intermediate and long acting insulin was withdrawn. During this time, blood glucose was controlled with multiple daily injections of soluble insulin, with the last injection being given between noon and 1300h on the day of the study. Subjects were admitted to the clinical investigation ward at 1700h and an intravenous cannula was sited in a vein in each arm; one for purposes of sampling and one for intravenous infusion. Subjects chose a meal from the ward menu (they received an identical meal on each of the 3 overnight visits) which was given at 1800h and were subsequently fasted until noon the next day.

Between 1800 and 0800h, a variable rate insulin infusion was administered. Blood glucose was determined every 15 minutes and the insulin infusion rate adjusted to maintain blood glucose at 5mmol/l: no glucose was administered during this period. In addition to glucose, samples were taken for growth hormone (15 minutely) and free insulin (every 30 minutes). Samples for non esterified fatty acids (NEFA), IGFBP-1, and ketones (AHA and  $\beta$ OHB) were taken every hour. During the overnight period and hyperinsulinaemic clamp a sampling chart was used for reference which is reproduced in Appendix 2.

### 2.3.5 *Hyperinsulinaemic Euglycaemic Clamp*

Between 0800 and 1200h the following morning, a 2-step hyperinsulinaemic euglycaemic clamp study, incorporating glucose and glycerol cold isotopes was performed as follows. At 0500h primed infusions ([6,6  $^2\text{H}_2$ ] glucose, 170mg intravenous bolus followed by continuous infusion, 1.7mg/min and  $^2\text{H}_5$  glycerol, 0.61mg/Kg bolus followed by 0.15mg/kg/min continuous infusion) were commenced and continued for the duration of the study. Between 0800h, and 1000h, step 1 of the hyperinsulinaemic clamp was performed (insulin bolus 3.5mU/Kg followed by insulin infusion 0.75mU/Kg/min). Between 1000h and 1200h, step two (insulin bolus 7mU/Kg followed by insulin infusion 1.5mU/Kg/min) of the hyperinsulinaemic clamp was performed. During the hyperinsulinaemic clamp, blood glucose was determined every 5 minutes and maintained at 5mmol/l using a variable rate 20% dextrose infusion. The 20% dextrose was enriched with 700mg [6,6  $^2\text{H}_2$ ] glucose per 500ml bag to prevent marked decreases in blood tracer enrichment and consequent calculation errors. Serum enrichment of [6,6  $^2\text{H}_2$ ] glucose and  $^2\text{H}_5$  glycerol were determined every 5 minutes during each of 3 steady state period (baseline 0730 to 0800h, step one 0930 to 1000h and step two 1130 to 1200h).

Throughout the study period (1800h to 1200h), blood samples were taken regularly for determination of free insulin (30 minutes), IGFBP-1, NEFA and  $\beta$ -hydroxybutyrate (60 minutes). A sample was collected at 0800h for IGF-I and IGFBP-3 determination. Subjects remained supine throughout the study period and had similar sleep patterns during each night of observation.

### 2.3.6 *Laboratory Analysis*

Blood glucose concentrations were measured using 25 $\mu$ l whole blood samples on a Y.S.I. model 2300 stat plus analyser (YSI Incorporated, Yellow Springs, Ohio 45387 USA). The intra-assay coefficient of variation (CV) at 4.1 mmol/L is 1.5%. The equivalent inter-assay CV at this glucose concentration was 2.8%, and was 1.7% at 14.1

mmol/L. Plasma insulin concentrations were measured by ELISA, using a commercial kit (DSL, Tooting, London) according to the manufacturer's instructions. The sensitivity of this assay was 0.26 mU/L. Intra-assay imprecision (CV) was 4.4% at 10.3 mU/L and 5.1% at 35.8 mU/L. Equivalent inter-assay imprecision was, 8.7% and 2.9% respectively. Plasma (total) IGF-I concentrations were measured in ethanolic extracts by ELISA, (DSL, Tooting, London, UK). The sensitivity of this assay was 0.03 ng/mL. Intra-assay imprecision (CV) was 8.8% and 9.4% at 107 and 262ng/ml respectively. Equivalent inter-assay imprecision (CV) was 6.1% and 8.0% at 107 and 262ng/ml respectively, respectively. Plasma (total) IGFBP-1 levels were measured by ELISA, (DSL, Tooting, London, UK). The sensitivity of this assay was 0.25 ng/mL. Intra-assay imprecision (CV) was 6.1% at 7.0 ng/mL and 5.3% at 48.4 ng/mL. Equivalent inter-assay imprecision was, 10.4% and 5.1% respectively. Plasma IGFBP-3 levels were measured by ELISA, (DSL, Tooting, London). The sensitivity of this assay was 0.04ng/mL. Intra-assay imprecision (CV) was 4.9% and 2.8% at 5.2 and 34.7ng/ml respectively. Equivalent inter-assay imprecision (CV) was 9.7% and 1.9% at 5.2 and 34.7ng/ml respectively.

Plasma glycerol concentration was measured by a direct enzymatic colorimetric method (Randox Laboratories Ltd, County Antrim, UK), (inter assay CV 3.7%) and NEFA was measured by an enzymatic colorimetric method (Wako chemicals, Neuss, Germany), (inter assay CV 3.6%) using a Cobas Fara II autoanalyzer (Roche, Welwyn Garden City, U.K.).  $\beta$ -hydroxybutyric acid ( $\beta$ OHB) was measured using a standard enzymatic technique (Sigma Diagnostics,  $\beta$ -hydroxybutyric acid kit, Poole, Dorset, UK).

Glucose and glycerol enrichment were measured by gas chromatography mass spectrometry (GC-MS) using a Hewlett Packard 5971A MSD (Agilent Technologies, Berks, UK). Glucose enrichment was determined from deproteinised plasma using the methoxime-trimethylsilyl ether derivative (Shojaee-Moradie, Jackson et al. 1996). Glycerol was isolated from deproteinised plasma using ion exchange chromatography

and glycerol enrichment was determined using the tris-trimethylsilyl derivative (Wolfe 1992; Elia, Kahn et al. 1993). GC-MS analysis used electron impact ionisation with selected ion monitoring of the ions at mass/charge ratio (m/z) 319 and 321 for glucose and 205 and 208 for glycerol.

### 2.3.7 Calculations

#### 2.3.7.i IGF-I/IGFBP-3 Molar Ratio

The molar ratio of total plasma IGF-I to IGFBP-3 was calculated according to the molecular weights of IGF-I and IGFBP-3 according to the following formula, using a molecular weight of 33 –kDa for IGFBP-3 and 7.5 –kDa for IGF-I.

$$\text{Molar Ratio} = \frac{33 \times [\text{IGF-I (ng/ml)}]}{7.5 \times [\text{IGFBP-3 (ng/ml)}]}$$

#### 2.3.7.ii Glucose Infusion Rate

Total body glucose utilisation (M value, mg/Kg/min) was determined during the final 30 minutes of both steps of the hyperinsulinaemic clamp. The mean rate of 20% dextrose infusion was calculated during the final 30 minutes of each step of the hyperinsulinaemic clamp (MnRate) and converted from ml/hour to mg/kg/min using the formula:

$$\text{M value (mg/kg/min)} = \text{MnRate/60 (ml/min)} \times 0.2 \times 1000 \text{ (mg/ml)} / \text{weight (kg)}$$

#### 2.3.7.iii Insulin Clearance

$$\text{Insulin Clearance (ml.kg/min)} = \frac{\text{Insulin Infusion Rate (mU/kg/min)}}{\text{Mean plasma insulin concentration (mU/ml)}}$$



#### 2.3.7.iv Glucose and Glycerol Turnover

The enrichments of glucose and glycerol were expressed as the tracer/tracee ratio. The rates of appearance and disposal of glucose and glycerol were calculated using both the Steele and Mari models for the non-steady state modified for use with stable isotopes (Steele 1959; De Bodo, Steele et al. 1963; Mari 1992). The effective volume of distribution was assumed to be 143ml/kg for glucose and 230ml/kg for glycerol (Steele 1959; Mari 1992; Romijn, Coyle et al. 1993). The calculations were performed under the supervision of Drs Fariba Shojaee-Moradie and Margot Umpleby (Department of Diabetes and Metabolism, St. Thomas's Hospital, London, United Kingdom) using Microsoft Excel.

#### 2.3.8 Statistical Analysis

Where data were normally distributed they were analysed using parametric tests. Serum insulin, IGFBP-1, IGFBP-3 and  $\beta$ OHB were not normally distributed and were log transformed (base 10) to permit parametric testing. The square root of NEFA values was used for analysis. Glucose Ra and Rd and glycerol Ra values were log transformed (base e) prior to analysis.

Repeated measures ANOVA was used to confirm the overnight steady state period of euglycaemia (0300-0800h). During this period of time there was no variation in blood glucose concentration with time; nor any difference in blood glucose concentrations between the 3 different study nights. This defined period of euglycaemia was then used to compare differences in insulin requirements, insulin concentrations and ketone body concentrations between the three study nights.

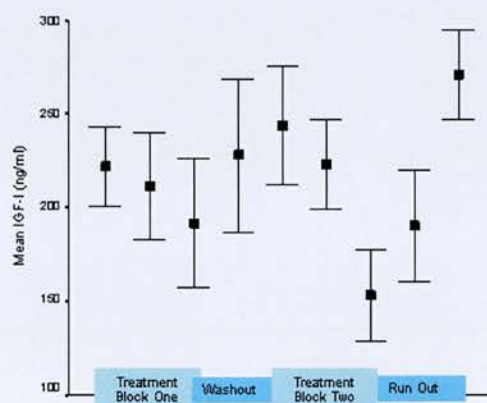
Comparisons of means of the 3 study nights were made using Students *t*-tests for paired samples, with each treatment period being compared with baseline. Differences in glucose and glycerol turnover were analysed using repeated measures ANOVA across time, with each treatment night being compared against the baseline night. SPSS version 10 (SPSS, Chicago, IL, USA) was used for all statistical analysis. *p* values less than 0.05

(two tailed) were considered statistically significant. Data are expressed as mean  $\pm$  SEM unless otherwise stated.

## 2.4. Results

### 2.4.1 Randomisation and Carry Over Effect

4 subjects (2M) received 5mg B2036-PEG (S5) first and 3 (1M) 10mg B2036-PEG (S10) first (Table 3). There was no carry-over effect observed; IGF-I levels were similar to those at baseline after treatment block 1, prior to treatment block 2, regardless of the order in which subjects received B2036-PEG; IGF-I concentration  $223.5 \pm 23.9$  ng/ml before treatment block 1 and  $238.0 \pm 30.1$  ng/ml before treatment block 2 ( $p=0.7$ ), (Figure 6).



**Figure 6: IGF-I Concentrations Throughout the Study**

Total IGF-I concentrations throughout the study shown chronologically rather than by dose. Error Bars represent 1 SEM.

### 2.4.2 Adverse Events

No serious adverse events were reported during the study. 2 subjects reported local swelling at the injection site, which spontaneously resolved within 2 hours. There were no episodes of severe hypoglycaemia reported by any subject during any part of the study protocol.

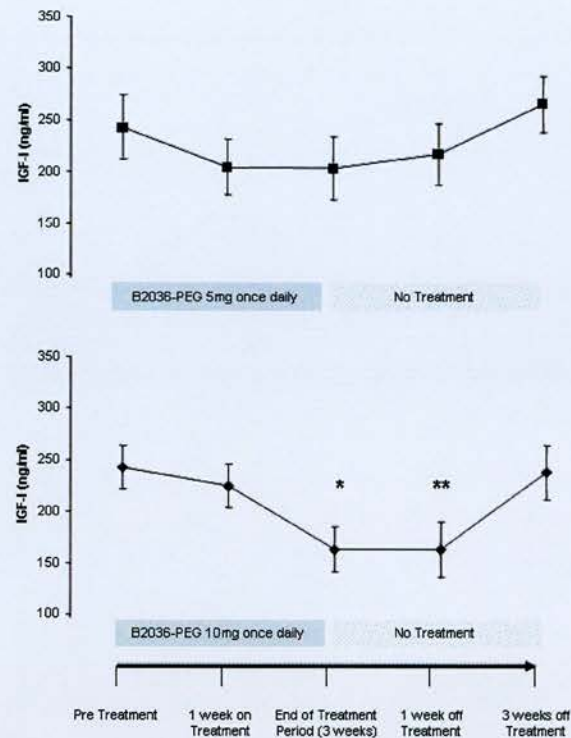


### 2.4.3 IGF-I and IGF Binding proteins

Effects of treatment with B2036-PEG on concentrations of IGF-I and IGFBP-3 are summarised in Table 4. Treatment with 5mg B2036-PEG tended to lower IGF-I concentration but this did not reach significance (Figure 7, Table 4). Treatment with 10mg B2036-PEG led to a 30% reduction ( $p=0.01$ ) in IGF-I concentrations after 3 weeks of treatment (Figure 7, Table 4). IGFBP-3 tended to be lower following treatment with B2036-PEG; ( $3.0 \pm 1.2$  mcg/ml at baseline vs  $2.9 \pm 0.3$  following S5 and  $2.7 \pm 0.3$  following S10), however this did not reach significance (Table 4). Similarly, the IGF-I/IGFBP-3 molar ratio tended to be lower following treatment with B2036-PEG; ( $0.48 \pm 0.05$  at baseline vs  $0.39 \pm 0.05$  following S5 vs  $0.39 \pm 0.08$  following S10) but did not reach statistical significance with either dose ( $p=0.09$ ) (Table 4).

Table 4. Effects of B2036-PEG on IGF-I and IGFBP-3  
(\* $p<0.05$  vs pre treatment)

	Pre-Treatment	5mg B2036-PEG	10mg B2036-PEG
IGF-I (ng/ml)	$223.5 \pm 23.9$	$183.8 \pm 38.1$	$154.6 \pm 28.1$ *
% Change in IGF-I	0	$-16.3 \pm 11.3$	$-36.2 \pm 7.3$
IGFBP-3 ( $\mu$ g/ml)	$3.01 \pm 0.1$	$2.94 \pm 0.3$	$2.69 \pm 0.3$
IGF-I/IGFBP-3 Molar Ratio	$0.48 \pm 0.05$	$0.39 \pm 0.05$	$0.39 \pm 0.08$

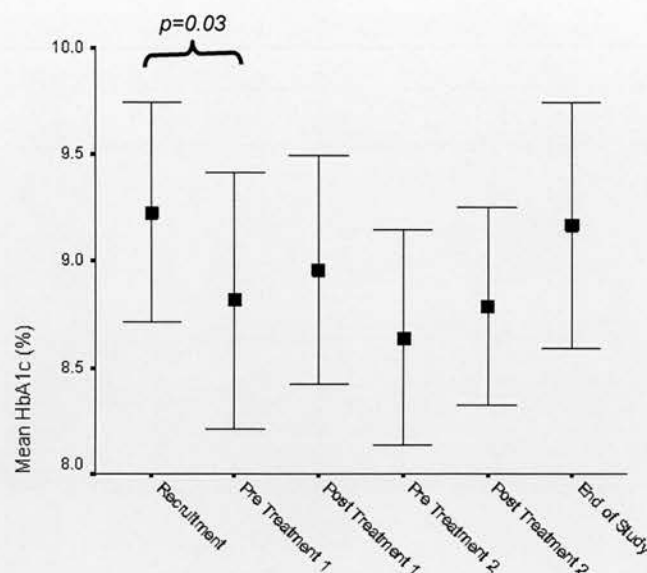


**Figure 7: Dose Effects on IGF-I Concentrations**

Upper Panel (black squares) 5mg Lower Panel (black diamonds) 10mg. Error Bars represent +/- 1 SEM \*  $p=0.004$  vs Pre Treatment \*\*  $p=0.01$  vs Pre Treatment

#### 2.4.4 HbA1c

HbA1c was measured regularly throughout the study in a central laboratory. By the end of the run-in period, HbA1c levels (%) had fallen from  $9.2 \pm 0.5$  to  $8.8 \pm 0.6$  & ( $p=0.03$ ). Thereafter it remained relatively constant and drifted back up again towards the end of the study (Figure 8).



**Figure 8:** *HbA1c throughout the study*

Mean HbA1c represented chronologically throughout the study period. Error bars represent 1 SEM

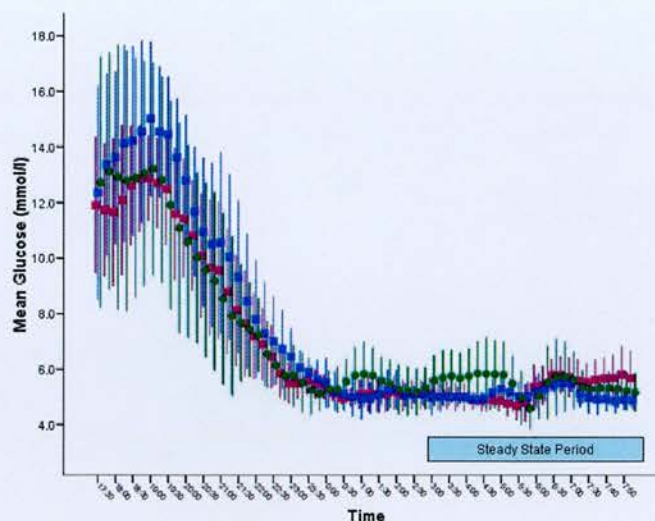
#### 2.4.4.i Overnight Steady State Euglycaemia

The overnight steady state period of euglycaemia (5mmol/l) was defined between 0300 and 0800h. During this time period, there was no difference across time, within or between individuals, on each of the 3 study nights (Table 5). This period was established using a repeated measures ANOVA model. Blood glucose concentrations on the 3 nights are shown in Figure 9.

#### 2.4.4.ii Overnight Insulin Requirements

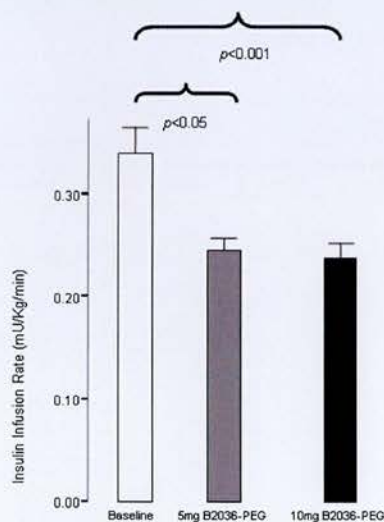
During the steady state period (0300-0800h) of euglycaemia (5mmol/l), there was no significant difference in blood glucose levels on the three study nights, but mean insulin infusion rates (mU/Kg/min) were reduced following treatment with B2036-PEG ( $0.35 \pm 0.03$  at baseline vs  $0.25 \pm 0.01$  ( $p=0.02$ ) following S5 and  $0.35 \pm 0.03$  at baseline vs  $0.24 \pm 0.01$  ( $p=0.004$ ) following S10 (Table 5, Figure 10).





**Figure 9: Overnight blood glucose profiles**

Mean blood glucose concentrations during the overnight insulin infusion for euglycaemia on the 3 study nights at baseline (pink), following treatment with 5mg (blue) and 10mg (green) B2036-PEG for 3 weeks. Steady state period of euglycaemia (0300 to 0800h) denoted by the shaded box. Error bars represent 2 SEM.



**Figure 10: Overnight Insulin Requirements for Euglycaemia**

Overnight (0300-0800h) insulin requirements (mU/Kg/min) for euglycaemia (5mmol/l) at baseline (white bar), following 5mg B2036-PEG (grey bar) and following 3 weeks 10mg B2036-PEG (black bar). \*  $p<0.05$  \*\* $p<0.001$



#### 2.4.4.iii Plasma insulin and IGFBP-1 concentrations

There were no differences in plasma free insulin levels during steady state euglycaemia on the three study nights (Table 5) and although corresponding reductions in derived insulin clearance were observed, they were not significant (Table 5). Plasma IGFBP-1 concentrations were increased following treatment with 10mg B2036-PEG (Table 5).

*Table 5. Effects of B2036-PEG on Overnight Glucose and Lipid Metabolism during Steady State Euglycaemia (0300 to 0800h)*

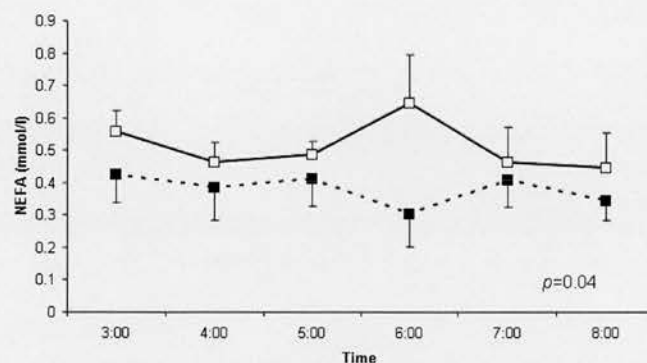
\* p<0.05, \*\*p<0.005 vs baseline

	Pre-Treatment	Following 5mg B2036-PEG	Following 10mg B2036-PEG
Glucose (mmol/l)	5.3 ± 0.07	5.1 ± 0.04	5.5 ± 0.09
Insulin Requirement (mU/Kg/min)	0.34 ± 0.03	0.25 ± 0.01 *	0.24 ± 0.02 **
Plasma Insulin (mU/l)	21.8 ± 2.4	19.3 ± 1.4	18.3 ± 1.6
Insulin Clearance Rate (ml.kg/min)	14.1 ± 1.5	14.2 ± 1.1	12.7 ± 1.3
NEFA (mmol/l)	0.51 ± 0.04	0.52 ± 0.04	0.38 ± 0.04 *
β-Hydroxybutyrate (mmol/l)	0.31 ± 0.04	0.25 ± 0.05	0.15 ± 0.02 **
IGFBP-1 (ng/ml)	41.5 ± 4.5	45.5 ± 7.5	55.4 ± 4.4 *

#### 2.4.4.iv Non esterified fatty acids and β- hydroxybutyrate

Treatment with S10 led to a significant reduction in mean overnight NEFA concentrations (Figure 11), ( $0.51 \pm 0.04$  mmol/l before treatment vs  $0.38 \pm 0.04$  following S10, p=0.03) but not during the hyperinsulinaemic clamp. Overnight mean

$\beta$ OH levels were also reduced following S10 ( $0.31 \pm 0.04$  mmol/l before treatment vs  $0.15 \pm 0.02$  following S10,  $p=0.001$ ).



**Figure 11: Effects of B2036-PEG on overnight NEFA levels**

Mean NEFA concentrations (mmol/l) during overnight (0300-0800h) steady state euglycaemia (5mmol/l) at baseline (white squares, solid line) and following 3 weeks 10mg B2036-PEG (black squares, broken line). Error bars represent 1 SEM.

#### 2.4.5 Hyperinsulinaemic Euglycaemic Clamp

Data from the hyperinsulinaemic clamp are summarised in Table 6.

##### 2.4.5.i Glucose and insulin concentrations

There was no difference between occasions in glucose or insulin concentrations during either step of the hyperinsulinaemic clamp (Table 6).

##### 2.4.5.ii Glucose infusion rate

Glucose infusion rate (mg/mg/min) did not change during either step of the hyperinsulinaemic clamp following treatment with either dose of B2036-PEG (Table 6).

##### 2.4.5.iii Endogenous hepatic glucose production

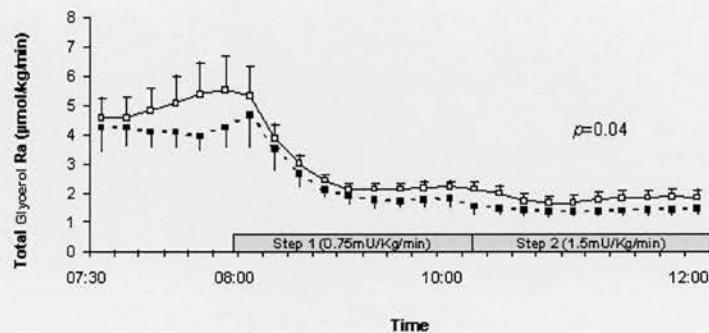
There was no change in endogenous hepatic glucose production (endoRa) at baseline (0730-0800h) or during either step of the hyperinsulinaemic clamp following treatment with B2036-PEG (Table 6).

#### 2.4.5.iv Peripheral glucose utilisation

There was no change in peripheral glucose utilisation (Rd) at baseline (0730-0800h) or during either step of the hyperinsulinaemic clamp following treatment with B2036-PEG (Table 6).

#### 2.4.5.v Glycerol turnover

Treatment with S10 led to reductions in glycerol turnover across the whole study period, but differences were not detected when each steady state was considered separately (Figure 12). Treatment with S5 had no effect on glycerol turnover.



**Figure 12: Effects of B2036-PEG on Glycerol Turnover**

Basal glycerol Turnover (0730 to 0800h) and during the 2-step hyperinsulinaemic euglycaemic clamp at baseline (open squares, solid line) and following treatment with S10 (black squares, dotted line).

Table 6. *Data from the Hyperinsulinaemic Clamp*

Effects of B2036-PEG on insulin sensitivity and lipid metabolism during the two-step hyperinsulinaemic clamp study. Baseline values were determined 0730-0800h. Step one (0.75mU/Kg/min) was performed from 0800-1000h and step 2 (1.5mU/Kg/min) from 1000-1200h.

		Pre-Treatment	Following 5mg B2036-PEG	Following 10mg B2036-PEG
Glucose (mmol/l)	Step One	4.9 ± 0.02	5.0 ± 0.03	4.9 ± 0.03
	Step Two	5.0 ± 0.06	5.0 ± 0.06	4.9 ± 0.04
Plasma Insulin (mU/l)	Step One	54.4 ± 4.4	43.8 ± 5.9	53.1 ± 4.6
	Step Two	97.9 ± 5.3	98.4 ± 12.6	107.4 ± 7.2
Glucose Infusion Rate (mg/kg/min)	Step One	3.0 ± 0.4	2.6 ± 0.3	3.1 ± 0.5
	Step Two	7.9 ± 0.6	7.0 ± 0.9	7.8 ± 0.7
	Baseline	9.6 ± 1.6	9.8 ± 0.8	8.5 ± 1.3
Glucose Ra (μmol/Kg/min)	Step One	4.1 ± 0.9	3.6 ± 0.6	2.6 ± 0.9
	Step Two	2.8 ± 1.8	2.1 ± 0.7	2.1 ± 2.3
	Baseline	9.2 ± 1.3	10.2 ± 0.7	9.7 ± 1.0
Glucose Rd (μmol/Kg/min)	Step One	22.1 ± 2.8	18.9 ± 2.1	22.1 ± 3.7
	Step Two	50.1 ± 4.6	42.1 ± 5.1	46.1 ± 6.8
Glycerol Production Rate (μmol/Kg/min)	Baseline	5.0 ± 0.9	3.6 ± 0.4	4.2 ± 0.5
	Step One	2.2 ± 0.2	2.5 ± 0.4	1.8 ± 0.2
	Step Two	1.1 ± 0.3	2.4 ± 0.5	1.4 ± 0.2
NEFA (mmol/l)	Step One	0.21 ± 0.05	0.26 ± 0.04	0.16 ± 0.02
	Step Two	0.04 ± 0.01	0.07 ± 0.03	0.02 ± 0.00

## 2.5. Discussion

Growth hormone has been implicated in the increased overnight insulin requirements seen in subjects with Type 1 Diabetes during puberty and in the pathogenesis of the dawn phenomenon. These data support this hypothesis, as 3 weeks' treatment with both 5 and 10mg B2036-PEG led to significant reductions in overnight insulin requirements. The extent to which these changes are mediated by suppression of growth hormone's known effects on lipolysis and NEFA generation, or by direct effects on peripheral glucose utilisation by muscle, or suppression of hepatic glucose production, remains uncertain. Increased overnight rates of lipolysis in young adults with Type 1 Diabetes, which are not corrected by a preceding period of euglycaemia has been reported, and may be related to increased growth hormone secretion (Hagstrom-Toft, Bolinder et al. 1997). Studies using the nicotinic acid analogue acipimox, which inhibits lipolysis, leading to reduced plasma NEFA levels, have demonstrated enhancement of insulin sensitivity in young, non-obese adults with T2DM (Fulcher, Catalano et al. 1992; Walker, Agius et al. 1993). A recent study has shown that withdrawal of growth hormone treatment in growth hormone deficient adults results in a decrease in glycerol and NEFA production rate, measured using stable isotopes, and an increase in insulin sensitivity (Gibney, Healy et al. 2003). Use of acipimox to prevent the rise in NEFA following growth hormone administration to growth hormone deficient subjects prevents the subsequent decline in insulin sensitivity (Nielsen, Moller et al. 2001). The data presented in this chapter data support a role of growth hormone in the regulation of lipolysis overnight, as blockade with the higher dose of B2036-PEG resulted in reduced NEFA and  $\beta$ OHB levels overnight and glycerol turnover rate during the clamp study. However, reduction in overnight insulin requirements for euglycaemia were also observed with the 5mg dose of B2036-PEG, yet NEFA levels,  $\beta$ OHB levels and glycerol turnover rates were not different from the baseline study. Given that the lower dose of B2036-PEG did not alter overnight NEFA or  $\beta$ OHB levels, or glycerol production rate,

it is possible that there is some direct effect of growth hormone blockade on hepatic insulin sensitivity.

The observed reductions in insulin requirement for euglycaemia were accompanied by a reduction in plasma insulin levels that was non-significant and was not accompanied by a discernible change in insulin clearance. It has previously been reported in adolescents with Type 1 Diabetes that overnight growth hormone concentrations are inversely related to overnight insulin requirements and directly related to insulin clearance (Acerini, Cheetham et al. 2000). Growth hormone blockade is unlikely to affect overnight insulin requirements through changes in IGF-I as there was no effect on IGF-I concentrations with the lower dose (although this may reflect the small sample size). Contrastingly, with S10, there was a reduction in IGF-I concentrations. Reductions in IGF-I were accompanied by non-significant changes in IGFBP-3 and a reduction in the IGF-I/IGFBP-3 molar ratio, suggesting a possible reduction in free, or bio available IGF-I. Increases, rather than decreases in IGF-I have previously been shown to reduce overnight insulin requirements, both directly (Crowne, Samra et al. 1998), and secondary to reductions in growth hormone secretion (Acerini, Harris et al. 1998). These data suggest that growth hormone blockade has effects on overnight insulin sensitivity that are independent of IGF-I, with an indirect effect on lipolysis seeming the most likely explanation, given the observed effects on lipolysis.

The reduction in IGF-I concentrations following growth hormone blockade may contribute to the failure to observe any changes in insulin sensitivity during the hyperinsulinaemic clamp the following morning, as IGF-I has been directly implicated both in hepatic and peripheral insulin sensitivity during a hyperinsulinaemic clamp. Direct effects of suppression of IGF-I concentrations on insulin sensitivity may therefore have ameliorated any beneficial effects of the indirectly beneficial effects of growth hormone blockade. Growth hormone blockade with B2036-PEG is prolonged and has been reported not to cause rebound stimulation of GH release via the hypothalamic-



pituitary axis (Thorner, Strasburger et al. 1999). As such, pharmacological blockade of the growth hormone receptor should have been maintained throughout the clamp period and NEFA were rapidly suppressed by insulin during step 1 of the hyperinsulinaemic clamp on all occasions; yet greater suppression of glycerol turnover following S10 was evident throughout the hyperinsulinaemic clamp. However, despite the greater suppression of glycerol production rate, insulin sensitivity in respect of either hepatic glucose production, or peripheral glucose utilisation remained unchanged.

This work would have been strengthened by the inclusion of placebo control data. In addition, there is always the possibility that changes in insulin action overnight might be secondary to a non-specific improvement in diabetes control. However this would not explain the lack of effect on insulin sensitivity during the clamp the following morning. The sample size of 7 subjects should have provided 80% power with 5% significance to detect a 30% change in endogenous hepatic glucose production during step one of the hyperinsulinaemic clamp. A larger sample size may have detected effects on insulin sensitivity of lesser magnitude.

Persisting IGF-I deficiency, particularly following S10 may explain the absence of change in insulin stimulated glucose disposal during the morning hyperinsulinaemic clamp. Recent studies have demonstrated that variation in free IGF-I levels following exogenous administration of rhIGF-I may be an important determinant of insulin sensitivity (Frystyk, Hussain et al. 1997). Unfortunately, in this study, there was insufficient blood available to allow determination of free IGF-I. Despite reports of decreased type 1 IGF receptor expression in the liver (Caro, Poulos et al. 1988), rhIGF-I administration leads to reductions in hepatic glucose production rate and increased peripheral glucose uptake (Acerini, Harris et al. 1998; Carroll, Christ et al. 2000; Cusi and DeFronzo 2000). The effects of IGF-I on adipose tissue in humans remains unclear, as human adipocytes do not express the type 1 IGF receptor (Frick, Oscarsson et al. 2000). In vitro studies have demonstrated that incubation of adipocytes with IGF-I

stimulates glucose uptake and lipogenesis, but these insulin-like effects are likely to be mediated via the insulin receptor (Siddals, Westwood et al. 2002). Thus reduced IGF-I levels following S10 could counteract any effects of growth hormone blockade and suppression of lipolysis on insulin sensitivity through direct effects at the liver and via the type 1 IGF receptor in other tissues such as muscle.

Alternatively, failure to observe an increase in insulin sensitivity during the hyperinsulinaemic clamp may reflect the method used to quantify insulin sensitivity rather than a true absence of change. Treatment with S10 led to a suppression of lipolysis overnight, with reductions in concentrations of free fatty acids. NEFAs are known to cause insulin resistance by the competitive inhibition of glucose transport into the cell by GLUT4 (Shulman 2000). The overnight insulin infusion for euglycaemia, is, by its very nature, physiological, with S10 treatment leading to an increase in insulin sensitivity. However, even during the first step of the hyperinsulinaemic clamp, doses of insulin are used which lead to plasma insulin concentrations which greatly exceed physiological levels; around 50mU/l (approximately 300pmol/l). If specific growth hormone blockade affects insulin sensitivity predominantly by its effect on lipolysis, which is exquisitely sensitive to insulin, as soon as the hyperinsulinaemic clamp protocol is commenced, any effect mediated via lipolysis will immediately be ameliorated by the resultant supra-physiological concentrations of insulin. The hyperinsulinaemic clamp technique, although the current 'gold standard' methodology for the physiological assessment and quantification of insulin sensitivity, was developed for the study of individuals with insulin resistance and impaired glucose tolerance, in whom plasma concentrations of insulin are chronically elevated and it may not be the ideal tool for the study of individuals with type one diabetes.

## 2.6. Conclusion

These data support the importance of overnight growth hormone, lipolysis and NEFA modulation in the pathogenesis of the dawn phenomenon in Type 1 Diabetes, although

they suggest that IGF-I concentrations may be the more relevant determinant of insulin sensitivity the next morning as determined by a hyperinsulinaemic euglycaemic clamp. The therapeutic potential of growth hormone inhibition with B2036-PEG, which is safe and well tolerated, may therefore depend on the relative degree of reduction in IGF-I concentrations. In this respect, there were observed reductions in overnight insulin requirement with S5, without compromising IGF-I concentrations, although, with less effect on lipolysis than observed with S10: data suggesting that doses of B2036-PEG could be titrated in order to achieve optimal glycaemic control.

It would be extremely interesting to expand the overnight study of glucose turnover to utilise cold isotope methodology throughout the overnight period of euglycaemia, before and after treatment with a growth hormone antagonist in order to further evaluate basal glucose turnover with physiological concentrations of insulin.

This study investigated the effects of specific growth hormone blockade on overnight insulin sensitivity and lipolysis in a small group of young adults with type one diabetes. The adjunctive therapy was well tolerated for a 3-week period with no serious side effects and led to reductions in overnight insulin sensitivity, which appear to be secondary to an effect on lipolysis. Large-scale clinical trials of at least 6 months of therapy would be needed in order to ascertain clinically relevant effects on HbA1c as well as evidence of safety and tolerability. However, specific growth hormone blockade in this vulnerable subgroup of patients may be of benefit in order to optimise glycaemic control during adolescence, thereby minimising the risk of the development of complications later in life. Further short-term physiological studies looking at the effects of specific growth hormone blockade on glomerular filtration rate and urinary albumin excretion rates would be of interest.

# **Chapter 3: THE EFFECTS OF rhIGF-I/rhIGFBP-3 COMPLEX ON RENAL PHYSIOLOGY IN Type 1 Diabetes**

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## **3.1. Introduction**

This chapter describes a study undertaken to evaluate a potential role for manipulation of the growth hormone – IGF-I axis in the risk of nephropathy development in young people with Type 1 Diabetes. Recent data from the Oxford Regional Prospective Study (ORPS) comparing microalbuminuria (MA) positive cases with MA negative controls matched for age, sex, puberty stage and duration of diabetes suggest that cases have lower total IGF-I concentrations, increased overnight growth hormone secretion and also increased glomerular filtration rate (GFR) 5 years from diagnosis (prior to the development of MA) than controls (Amin, Turner et al. 2005; Amin, Williams et al. 2005). Increased secretion of growth hormone secondary to low circulating levels of IGF-I may stimulate increased paracrine production of IGF-I locally within the kidney and promote microangiopathic changes (Segev, Landau et al. 1997).

There is compelling evidence from rodent models of diabetes that nephropathy correlates directly with IGF-I expression within the kidney (Flyvbjerg 2000). An experimental model of Type 1 Diabetes in mice has abnormalities of the growth hormone - IGF-I axis similar to those seen in humans (Landau, Segev et al. 2000) and administration of specific growth hormone antagonists (GHAs) to mice with nephropathy leads to a reduction in renal expression of IGF-I, renal size, GFR, effective renal plasma flow (ERPF) and urinary albumin excretion (Flyvbjerg, Bennett et al. 1999; van Neck, Dits et al. 2000). Suppression of growth hormone secretion, or inhibition of its actions, may be a possible therapeutic adjunct in those at risk of nephropathy.

The study described in this chapter examined the effects of restoration of IGF-I concentrations using rhIGF-I/rhIGFBP-3 complex (Somatokine, SK) on overnight

growth hormone secretion and insulin requirements, GFR, ERPF and urinary excretion of albumin, retinol binding protein and growth hormone dependent peptides (IGF-I and IGFBP-3) in normalbuminuric young adults with Type 1 Diabetes.

### 3.2. Methods

#### 3.2.1 *Study Design*

The study was of randomised, double blinded, placebo-controlled crossover design. Each subject received two, 7-day treatment blocks of rhIGF-I/rhIGFBP-3 complex (0.4mg/kg once daily via subcutaneous injection, equivalent to IGF-I 80mcg/kg/day) and placebo in random order. There was a 2-week run in period and the treatment blocks were separated by a 2-week washout period. Ethical approval was obtained from the Cambridge Local Regional Ethics Committee and all studies were performed in the Wellcome Trust Clinical Research Facility (WTCRF) at Addenbrooke's Hospital. Written, informed, consent was obtained from all subjects, prior to commencement of the study protocol.

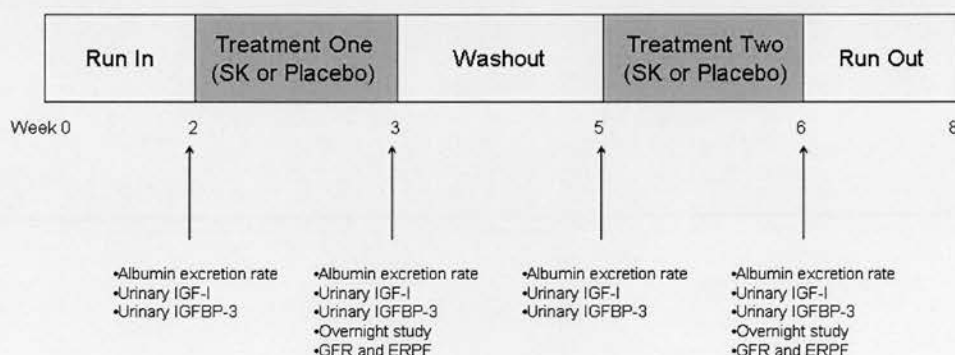
#### 3.2.2 *Subjects*

Seven subjects were recruited from the young adult diabetes clinic at Addenbrooke's Hospital, Cambridge. Details of individual subjects are given in Table 7. Three were male and median (Range) age was 21 (19-24) years with median (Range) HbA1c 8.9 (7.9-11.7) %. All were post pubertal, with Type 1 Diabetes of at least 2 years' duration, and C-peptide negative. All had normal renal, hepatic and thyroid function and a normal full blood count. None had any evidence of microalbuminuria or retinopathy. All were treated with a combination of short and long acting insulins two to four times per day (median insulin dose 0.9u/Kg/24hrs (range 0.5-1.1)). Seven subjects completed the study protocol, but 1 subject (subject 7) was omitted from the analyses as she neglected to omit her long acting insulin preparation prior to the second study night. One subject (subject 3, female) withdrew from the study following screening. The study timetable and investigations are shown in Figure 13.

Table 7. Subject Characteristics at Recruitment							
Subject ID	Treatment Order #	Insulin Dose (u/kg)	Regimen	Age (yr)	BMI (kg/m2)	Sex	HbA1c (%)
1	1	1.28	MIT	18.7	27.1	M	8.9
2	2	0.66	MIT	23.8	23.2	M	7.1
4	2	0.86	MIT	22.1	28.2	F	11.7
5	1	0.65	MIT	23.3	22.2	F	8.1
6	2	0.91	MIT	19.5	26.7	F	9.8
7 <sup>a</sup>	1	0.84	MIT	20.2	24.8	M	9.5
8	1	0.82	MIT	20.6	27.3	F	8.5

# 1: Treatment order Placebo followed by rhrhIGF-I/rhIGFBP-3 complex. 2 rhrhIGF-I/rhIGFBP-3 complex followed by Placebo. MIT: multiple injection therapy.

<sup>a</sup> Subject 7 was excluded from analysis due to failure to omit long acting insulin prior to one of the study nights.



**Figure 13: Study Schedule**

Timetable of treatments and investigations. The overnight study performed at the end of each treatment block comprises an overnight variable rate insulin infusion to achieve euglycaemia (5mmol/l). SK: Somatokine rhIGF-I/rhIGFBP-3 complex.



### 3.2.2.i Run in Period

The study commenced with a 2-week run in period. During this time, subjects were encouraged to perform home glucose monitoring at least 3 times daily and insulin dose was optimised.

### 3.2.2.ii Treatment Blocks

Each subject received 7 days treatment with placebo and rhIGF-I/rhIGFBP-3 complex (Somatokine, SK) (0.4mg/kg once daily via subcutaneous injection) in random order, separated by a 2-week washout period (Figure 13). Both subjects and investigator were blinded as to treatment. For the purposes of safety a member of the diabetes team (Dr Kevin Yuen) who was not otherwise involved with the study liaised with subjects during each treatment block regarding adjustment of insulin dose whilst receiving rhIGF-I/rhIGFBP-3 complex in order to minimise risk of hypoglycaemia. Subjects were advised to reduce their total insulin dose by 30% at the beginning of each treatment block, regardless of treatment. For the remainder of the seven days, insulin dose was titrated according to home blood glucose monitoring targets as outlined in Appendix 1.

Subjects were advised to administer the injection at the same time (1800h) each day, into the left thigh. Subjects were issued with 7 days' supply of rhIGF-I/rhIGFBP-3 complex or placebo which was stored frozen at -20 °C and removed 60 minutes prior to injection. Vials were then left at room temperature to allow the contents to defrost and come up to room temperature prior to injection.

### 3.2.3 24 hour Urine Collections

Subjects were asked to perform a total of four 24-hour urine collections throughout the study; one at the beginning and one at the end of each treatment block. Subjects were instructed to void their bladder on waking on the morning of the collection and subsequently to collect all urine into a sterile, untreated container. On waking the following morning, subjects were asked to completely empty their bladder, completing

the 24-hour collection. Volume was then determined and aliquots of urine centrifuged at 3000rpm for 5min to precipitate debris. Aliquots were stored at -80°C until analysed.

#### 3.2.4 *Overnight Variable Rate Insulin Infusion*

On the final day of each treatment block, subjects were admitted to the WTCRF for an overnight study. All long and medium acting insulin was withdrawn for 24 hrs prior to the overnight study. Subjects were admitted to the ward at 1730h and a cannula was sited in each antecubital fossa; one for the purposes of sampling and one for the purposes of infusion. A meal was given at 1800h (subjects were allowed to choose from a selection of sandwiches, baked potatoes etc and each subject received identical meals on both occasions).

Between 1800h and 0800h a variable rate insulin infusion for euglycaemia (5mmol/l) was performed with determination of blood glucose every 15 minutes. Samples were taken into lithium heparin tubes for determination of plasma growth hormone every 15 minutes and plasma IGF-I, IGFBP-1, IGFBP-3 and insulin concentrations every 60 minutes.

#### 3.2.5 *Estimation of GFR and ERPF*

Following the overnight period of euglycaemia, subjects underwent the following protocol to attempt estimation of GFR and ERPF using standard clearances of iothalamate and para-aminohippuric acid (PAH) respectively (Bell, Bombardt et al. 1994).

At 0700h infusions of iothalamate (bolus 20mg/kg followed by infusion rate 600mg/hr = 16µmol/min) and PAH (bolus 5mg/kg followed by infusion 750mg/hr) were commenced. At 0600h, subjects were asked to orally ingest approximately 1000ml of water in order to establish diuresis of the order of 10-25ml/min. If subjects were unable to comply with this, a similar volume of 0.9% saline was administered intravenously between 0700 and 0800h. After the initial hydration, subjects were asked to ingest

sufficient water orally in order to keep pace with urinary losses and thus maintain the diuresis.

After a 60 minute equilibration period, subjects were asked to empty their bladder completely and baseline plasma samples were taken into lithium heparin for determination of iothalamate and PAH. Subsequently plasma samples were taken every 10 minutes for determination of iothalamate and PAH and 3 accurately timed urine samples were collected and volume determined by the use of electronic weighing scales for determination of iothalamate and PAH concentration.

For valid use of standard clearance methodology adequate diuresis must be established so that residual bladder volume is of minimal significance. Diuresis was considered adequate if the calculated urinary excretion rate of iothalamate in an individual was within 20% either side of expected ( $16\mu\text{mol}/\text{min}$ , the infusion rate). Where this was not achieved, GFR was calculated from the continuous infusion clearance of iothalamate, from the mean of plasma iothalamate concentrations at 30, 60 and 90 minutes (see results section for further details). There is no alternative to standard clearance methodology for determination of ERPF.

$\text{Iothalamate Clearance} = \text{Infusion Rate} / \text{Plasma Concentration}$

GFR was subsequently corrected for body surface area and expressed /  $1.73\text{m}^2$

### *3.2.6 Laboratory Methods*

#### *3.2.6.i Quantitative assay techniques: plasma*

Blood glucose concentrations were measured using  $25\mu\text{l}$  whole blood samples on a Y.S.I. model 2300 stat plus analyser (YSI Incorporated, Yellow Springs, Ohio 45387 USA). The intra-assay imprecision (coefficient of variation, CV) at  $4.1\text{ mmol}/\text{L}$  was 1.5%. The equivalent inter-assay CV at this glucose concentration was 2.8%, and was 1.7% at  $14.1\text{mmol}/\text{L}$ .

Plasma insulin concentrations were measured by ELISA, using a commercial kit (DSL-UK Ltd, Upper Heyford, Oxon, UK) according to the manufacturer's instructions. The sensitivity of this assay was 0.26 mU/L. Intra-assay CV was 4.4% at 10.3 mU/L and 5.1% at 35.8 mU/L. Equivalent inter-assay CV was, 8.7% and 2.9% respectively.

Plasma (total) IGF-I concentrations were measured in ethanolic extracts by ELISA, (DSL-UK Ltd). The sensitivity of this assay was 0.03 ng/mL. Intra-assay CV was 8.8% and 9.4% at 107 and 262ng/ml respectively. Equivalent inter-assay CV was 6.1% and 8.0% at 107 and 262ng/ml respectively, respectively.

Plasma (total) IGFBP-1 levels were measured by ELISA, (DSL-UK Ltd). The sensitivity of this assay was 0.25 ng/mL. Intra-assay CV was 6.1% at 7.0 ng/mL and 5.3% at 48.4 ng/mL. Equivalent inter-assay CV was 10.4% and 5.1%, respectively.

Plasma IGFBP-3 levels were measured by ELISA, (DSL-UK Ltd). The sensitivity of this assay was 0.04ng/mL. Intra-assay CV was 4.9% and 2.8% at 5.2 and 34.7ng/ml respectively. Equivalent inter-assay CV was 9.7% and 1.9% at 5.2 and 34.7ng/ml respectively.

Plasma creatinine and iothalamate were measured by stable isotope dilution electrospray mass spectrometry-mass spectrometry. The creatinine assay has been reported previously (Lamb, Wood et al. 2005). Standards, samples, and controls (5µl) were diluted with 250µl of an aqueous internal standard mix (60µl 10mM iothalamate-d<sub>3</sub> and 20µl 10mM creatinine-d<sub>3</sub> per 100ml) directly into a 96 deep well (2ml) polypropylene plate (Semat International Ltd, St Albans, UK) using a Hamilton Micro Lab M (Hamilton, Carnforth, UK). Acetonitrile with 0.05% formic acid (250µl) was added to each well and the plate covered with a sealing mat, mixed, centrifuged, and loaded onto an CTC Analytics HTS PAL refrigerated autosampler (Presearch Ltd, Hitchin, UK) for MSMS analysis. Samples (2µl) were automatically introduced into a continuous solvent stream of acetonitrile:water (1:1) containing 0.025% formic acid flowing at 250µl/min (Agilent 1100 series). Chromatography, to minimise matrix ion suppression effects, was

performed on a Symmetry C8, 3.5 $\mu$ m 2.1x50mm column with guard (Waters Corporation, Watford, UK). MSMS was performed on a SCIEX API 4000 (Applied Biosystems, Warrington, UK) triple quadrupole MSMS with an electrospray source in positive ion multiple reaction monitoring (MRM) mode at 5250V and 250 $^{\circ}$ C. The interface heater was on and all other settings were optimized for the MRM transitions m/z 114.2/44.2 for creatinine, m/z 117.2/47.2 for creatinine-d<sub>3</sub>, m/z 614.7/360.9 for iothalamate, and m/z 617.7/363.9 for iothalamate-d<sub>3</sub>. Dwell time 150ms for each transition. Inject to inject time 3min. The inter-assay CVs for creatinine at 65 $\mu$ mol/l and iothalamate at 100 $\mu$ mol/l were 4.6% and 3.9%, respectively.

### 3.2.6.ii Quantitative assay techniques: urine

Urinary concentrations of IGF-I and IGFBP-3 were measured using commercially available ELISA kits (DSL, Tooting, London, UK). Prior to analysis urine was concentrated 10 fold (Centricon). For both peptides, the standard method was adapted to incorporate incubation of samples with the primary antibody for 18 hours at 4 $^{\circ}$ C.

Urine albumin and retinol binding protein were measured as previously described (Tomlinson, Dalton et al. 1990; Schultz, Dalton et al. 2000), except that urine dilutions and plating out were performed using a Hamilton Micro Lab Duo with a Micro Lab 500 (Hamilton, Carnforth, UK). Urine creatinine was measured by MSMS as above. 10 $\mu$ l of urine was automatically diluted 1:100 with deionised water (Hamilton Micro Lab Duo with a Micro Lab 500) and then further diluted 1:100 with creatinine-d<sub>3</sub> internal standard (20 $\mu$ l 10mM creatinine-d<sub>3</sub> per 200ml deionised water) directly into a 96 deep well polypropylene plate for MSMS analysis.

### 3.2.7 *Randomisation*

Randomisation was carried out by a third party who was not otherwise involved with the study or any of the participants. Subjects were randomised using a random number generator, and were stratified for gender in order to achieve equal proportions of both sexes in each treatment arm. Allocation of treatment arms was concealed from members

of the research team and study participants prior to randomisation. Treatment allocation was held in a password protected computer file and was revealed only once the study had been completed.

### 3.2.8 *Statistical Analysis*

Where data were normally distributed they were analysed using parametric tests. Serum insulin, IGFBP-1 and IGFBP-3 were not normally distributed and were log transformed (base 10) to allow parametric testing. All urinary data were log (10) transformed to allow parametric testing. For albumin excretion rate, urinary retinal binding protein, IGF-I and IGFBP-3, the change across each treatment block was calculated and then paired Students *t*-tests used to analyse differences in change within individuals.

A repeated measures ANOVA model incorporating subject ID and treatment as fixed factors was used to identify the overnight steady state period of euglycaemia (0200-0700h). During this time there was no difference in blood glucose across time either within or between individual subjects, or between placebo and rhIGF-I/rhIGFBP-3 complex.

Comparisons between means of variables collected during the 2 study nights were made using Students *t*-tests for paired samples, with rhIGF-I/rhIGFBP-3 complex being compared against placebo in each individual.

SPSS version 10 (SPSS, Chicago, IL) was used for all statistical analysis. *p* values less than 0.05 (two tailed) were considered statistically significant.



### 3.3. Results

Data are presented as mean (SD) or median (IQR) unless otherwise stated.

#### 3.3.1 *Randomisation*

4 subjects were randomised to receive placebo first and rhIGF-I/rhIGFBP-3 complex second and the remaining 3 received rhIGF-I/rhIGFBP-3 complex in their first treatment block and placebo in the second.

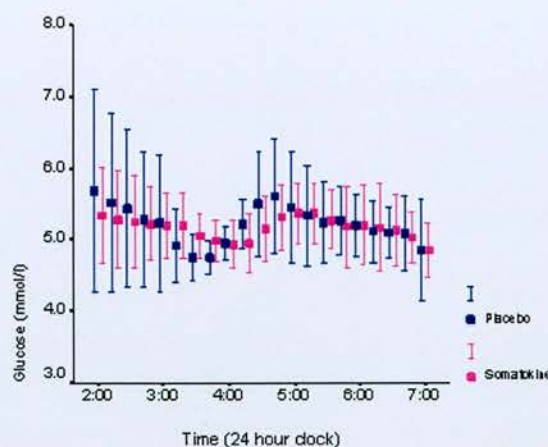
#### 3.3.2 *Adverse Events*

No serious adverse events occurred during the study. One subject reported mild bruising at the injection site while taking rhIGF-I/rhIGFBP-3 complex and three subjects complained of mild discomfort when injecting rhIGF-I/rhIGFBP-3 complex. Great care was taken to avoid hypoglycaemia, by reduction of insulin dose by around 30% while taking the active compound. There were no episodes of severe hypoglycaemia (defined as needing assistance from another individual) during the study, either in the rhIGF-I/rhIGFBP-3 complex or the placebo arm.

#### 3.3.3 *Overnight Steady State Period of Euglycaemia*

The overnight steady state period of euglycaemia was defined as between 0200 and 0700h. During this time, euglycaemia was maintained at 5mmol/l on both nights; with no significant difference in blood glucose across time on individual nights, or between nights, either within or between individuals (Figure 14).

Compared to placebo, subjects required less insulin overnight to maintain euglycaemia following seven days treatment with rhIGF-I/rhIGFBP-3 complex; 0.14 (0.1) vs 0.20 (0.2) mU/kg/min ( $p<0.04$ ) (Figure 15).

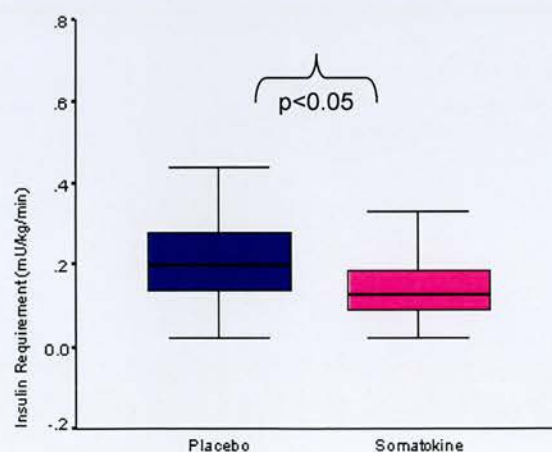


**Figure 14: Overnight Period of Euglycaemia**

Mean glucose concentrations (mmol/l) during steady state euglycaemia (5mmol/l) following placebo (blue) and rhIGF-I/rhIGFBP-3 complex (Somatokine) (pink). Error bars denote 2 SEM.

### 3.3.4 Growth Hormone Secretion

Following rhIGF-I/rhIGFBP-3 complex treatment, mean overnight growth hormone concentrations were lower following rhIGF-I/rhIGFBP-3 complex than following placebo; 2.6 (0.5) mU/l vs. 4.8 (0.5) ( $p < 0.04$ ).

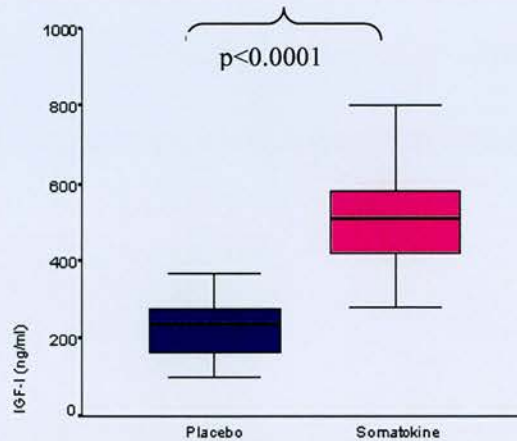


**Figure 15: Overnight Insulin Requirements**

Median (IQR) overnight (0200-0700h) insulin requirements for euglycaemia following placebo (blue) and rhIGF-I/rhIGFBP-3 complex (Somatokine) 0.4mg/kg/24hrs (pink).

### 3.3.5 IGF-I Concentrations

The mean overnight plasma concentration of IGF-I was significantly greater following rhIGF-I/rhIGFBP-3 complex treatment; 233 (81) vs 523 (154) ng/ml ( $p<0.0001$ ), (Figure 16).

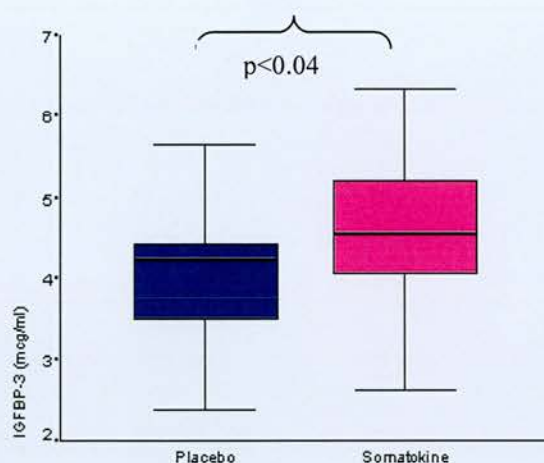


**Figure 16: IGF-I concentrations**

Median (IQR) overnight (0200 to 0700h) concentrations of IGF-I following seven days treatment with placebo (blue) and rhIGF-I/rhIGFBP-3 complex (Somatokine) 0.4mg/kg/24hrs (pink).  $p<0.001$

### 3.3.6 Concentrations of IGFBP-3

The mean overnight plasma concentration of IGFBP-3 was greater following rhIGF-I/rhIGFBP-3 complex treatment than following placebo; 4.5 (0.8) vs 4.0 (1.0) mcg/ml, ( $p<0.04$ ) (Figure 17).



**Figure 17: IGFBP-3 Concentrations**

Median (IQR) overnight (0200 to 0700h) concentrations of IGFBP-3 following seven days treatment with placebo (blue) and rhIGF-I/rhIGFBP-3 complex (Somatokine) 0.4mg/kg/24hrs (pink).

### 3.3.7 Plasma Creatinine Concentrations

Plasma creatinine concentration was lower following 7 days rhIGF-I/rhIGFBP-3 complex treatment than following placebo; 56.2 (12.8) vs 63.5 (15.0), ( $p < 0.02$ ).

### 3.3.8 Renal Physiology

In order to establish the validity of the standard clearance data for iothalamate and PAH, the excretion rate of iothalamate over the total time of urine collection was determined for each subject on each occasion. The expected excretion rate was  $16 \mu\text{mol/min}$  (equivalent to the infusion rate). Data were accepted if the calculated excretion rate fell within 20% either side of this, i.e. between  $12.8$  and  $19.2 \mu\text{mol/min}$  on both occasions. Excretion rates of iothalamate for individual subjects on each of the 2 occasions are shown in table 8. This was achieved in only 4 of the 7 subjects on both occasions, which almost certainly reflects error due to residual bladder volume due to an insufficient urine flow rate. This could explain both high and low excretion rates of iothalamate.

Due to the inadequate nature of the data from the standard clearance calculations, these were not included in analysis, and the steady state continuous infusion clearance of iothalamate was used to determine GFR as an alternative. Unfortunately there is no surrogate method for determination of ERPF and for this reason, no ERPF data are presented.

Subject	Iothalamate Excretion Rate ( $\mu\text{mol/min}$ )		Between 12.8 & 19.2 on both occasions?
	Study 1	Study 2	
1	17.4	13.4	Yes
2	14.0	9.9	No
4	16.4	14.5	Yes
5	13.4	15.1	Yes
6	40.6	13.9	No
7	12.1	13.5	No
8	18.9	14.5	Yes

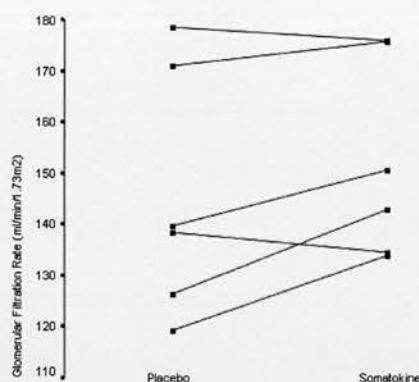
Table 8. *Individual Excretion Rates of Iothalamic Acid*

Excretion rates of iothalamate in individual subjects on each occasion. The expected excretion rate is  $16\mu\text{mol/min}$  and data were acceptable only if the actual excretion rate fell within 20% of this value (i.e. between 12.8 and  $19.2\mu\text{mol/min}$ ) on both occasions

### 3.3.8.i Glomerular Filtration Rate

There was no difference in glomerular filtration rate following treatment with rhIGF-I/rhIGFBP-3 complex when compared with placebo;  $151.8 (17.6)$  vs  $146.9 (22.2)$   $\text{ml/min/1.73m}^2$ . Overall, 4 subjects had an increase in GFR, and 2 a reduction (Figure 18).





**Figure 18: Individual Glomerular Filtration Rates**

Glomerular Filtration Rate in individual subjects following 7 day's treatment with placebo and rhIGF-I/rhIGFBP-3 complex (Somatokine) 0.4mg/kg/24hrs.

### 3.3.9 Urinary Albumin Excretion Rate

There was no difference in AER before and after treatment with either placebo or rhIGF-I/rhIGFBP-3 complex. There was no difference in the change in AER during treatment with placebo or rhIGF-I/rhIGFBP-3 complex (Figure 19). Individual subjects' urinary albumin excretion rates during the study are shown in Table 9.

### 3.3.10 Urinary Retinol Binding Protein

There was no difference between urinary concentrations of retinol binding protein following treatment with rhIGF-I/rhIGFBP-3 complex or placebo; 15.3 (3.7) vs 16.3 (7.0)  $\mu\text{g}/\text{mmol}$  creatinine. There was no difference in the change in urinary concentrations of retinol binding protein RBP between rhIGF-I/rhIGFBP-3 complex and placebo; +2.6 (5.7) following placebo and +4.9 (4.9)  $\mu\text{g}/\text{mmol}$  creatinine following rhIGF-I/rhIGFBP-3 complex.



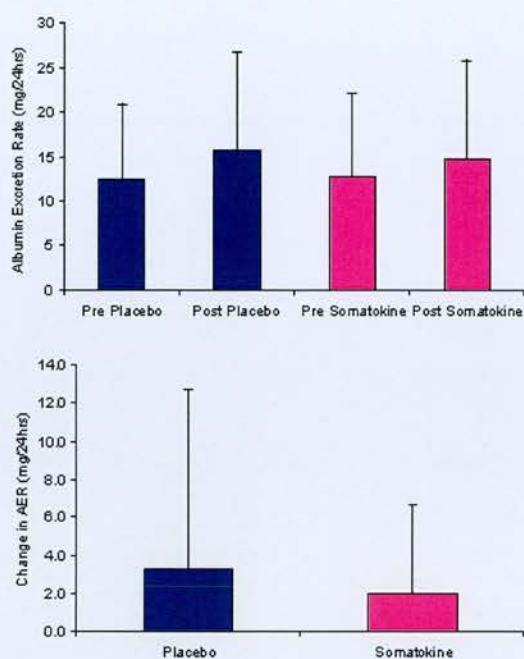
Subject	Placebo			rhIGF-I/rhIGFBP-3 complex		
	Pre	Post	Change	Pre	Post	Change
1	10.3	9.9	-0.4	7.9	10.4	2.5
2	5.8	28.5	22.7	11.4	13.7	2.3
3	8.9	7.6	-1.3	5.5	11.0	5.5
4	11.1	9.0	-2.0	5.3	8.5	3.1
5	18.8	13.1	-5.7	11.2	12.1	0.9
6	4.3	8.3	4.0	15.5	8.1	-7.4
7	28.2	34.1	5.9	32.3	39.5	7.2
Mean	12.5	15.8	3.3	12.7	14.8	2.0
SD	8.4	10.8	9.4	9.3	11.1	4.7

Table 9. *Albumin Excretion Rates*

Albumin excretion rate (mg/24hr) before and after seven days' treatment with placebo and rhIGF-I/rhIGFBP-3 complex 0.4mg/kg/day.

### 3.3.11 *Urinary Excretion of IGF-I and IGFBP-3*

There was no difference between urinary excretion of IGF-I before and after treatment with either placebo or rhIGF-I/rhIGFBP-3 complex (Table 10). Contrastingly, urinary concentration of IGFBP-3 was increased following treatment with rhIGF-I/rhIGFBP-3 complex but not following placebo (Table 10, Figure 20).



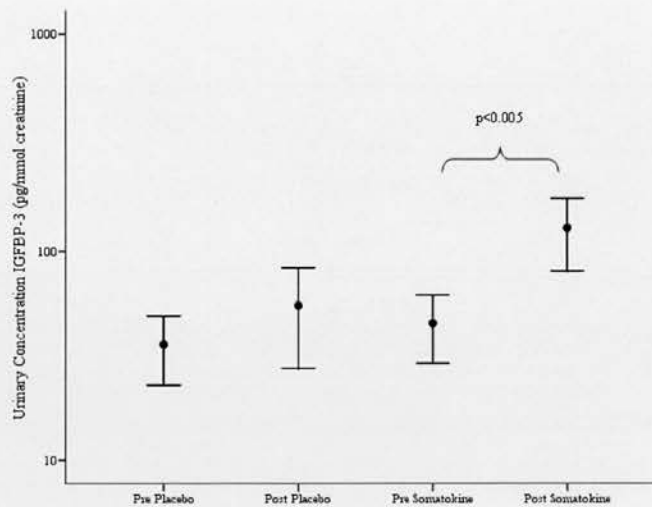
**Figure 19: Urinary Albumin Excretion Rates**

Effects of rhIGF-I/rhIGFBP-3 complex Treatment on Urinary Albumin Excretion Rate. A) AER before and after placebo (blue) and rhIGF-I/rhIGFBP-3 complex 0.4mg/kg/24hrs (pink) B) Change in AER over one week's treatment with placebo (blue) and rhIGF-I/rhIGFBP-3 complex 0.4mg/kg/24hrs (pink).

	Placebo		rhIGF-I/rhIGFBP-3 Complex	
	Pre	Post	Pre	Post
Urinary IGF-I (pg/ $\mu$ mol creatinine)	26.2 $\pm$ 8.5	33.1 $\pm$ 12.4	38.9 $\pm$ 15.9	38.4 $\pm$ 14.7
Urinary IGFBP-3 (mcg/mmol creatinine)	36.7 $\pm$ 14.3	56.1 $\pm$ 30.0	46.3 $\pm$ 17.8	128.8 $\pm$ 66.9 *

**Table 10. Effects of rhIGF-I/rhIGFBP-3 complex on Urinary Excretion of IGF-I and IGFBP-3**

Urinary concentrations of IGF-I and IGFBP-3 before and after treatment with placebo and rhIGF-I/rhIGFBP-3 complex 0.4mg/kg/day for seven days. \*  $p < 0.005$  compared with pre treatment.



**Figure 20: Urinary Excretion of IGFBP-3**

Effects of rhIGF-I/rhIGFBP-3 complex (Somatostatin) Treatment on urinary excretion of IGFBP-3 as detected by ELISA, adjusted for urinary creatinine concentration. Error Bars represent 95 % confidence intervals.

### 3.4. Discussion

Previously reported studies of 48-hours treatment with rhIGF-I/rhIGFBP-3 complex have reported increases in insulin sensitivity comparable to those seen following administration of rhIGF-I alone (Cheetham, Clayton et al. 1994; Acerini, Harris et al. 1998; Clemmons, Moses et al. 2000; Saukkonen, Amin et al. 2004). The data presented in this chapter confirm that the acute effects of treatment with rhIGF-I/rhIGFBP-3 complex are sustained over a seven day treatment period, with comparable increases in concentrations of IGF-I and IGFBP-3 and reductions in growth hormone secretion. Treatment was well tolerated, with no episodes of severe hypoglycaemia. The original hypothesis was that suppression of nocturnal growth hormone secretion might lead to reductions in GFR and urinary albumin excretion rates, however, there was no discernable change in GFR or albumin excretion rate following administration of rhIGF-I/rhIGFBP-3 complex. This may reflect differing effects of increases in IGF-I concentrations and suppression of growth hormone on renal filtration. It has been reported that administration of rhIGF-I (dose 60mcg/kg tds) leads to increases in

glomerular filtration rate that are apparent 6 hours following commencement of rhIGF-I therapy in healthy volunteers (Hirschberg and Kopple 1989; Hirschberg, Rabb et al. 1989; Hirschberg, Brunori et al. 1993; Hoogenberg, Sluiter et al. 1993; Hirschberg and Adler 1998). Similarly, administration of growth hormone leads to increases in GFR, but these are not apparent until three days after injection and are thought to be secondary to rises in IGF-I concentrations rather than a direct effect of growth hormone on the kidney (Hirschberg, Rabb et al. 1989). Previous studies of short and long-term administration of rhIGF-I alone to young people with Type 1 Diabetes reported no change in GFR (Cheetham, Holly et al. 1995; Acerini, Patton et al. 1997).

While there was no discernable effect on GFR, plasma creatinine concentrations were lower following rhIGF-I/rhIGFBP-3 complex. With no concomitant change in GFR, and no apparent change in renal tubular function, this may reflect lower protein turnover following rhIGF-I/rhIGFBP-3 complex treatment, secondary to the known anabolic and anti-catabolic effects of rhIGF-I (Mauras, Martinez et al. 2000). Detailed studies of protein turnover using stable isotopes would provide interesting additional insight into the mechanisms underlying this observation.

In a mouse model of diabetic nephropathy with abnormalities in the growth hormone - IGF-I axis similar to those seen in humans with Type 1 Diabetes, treatment with a specific growth hormone antagonist led to reductions in renal expression of IGF-I, combined with reductions in renal size, glomerular hyperfiltration and albumin excretion rates (Flyvbjerg, Bennett et al. 1999). However, in these studies circulating concentrations of IGF-I, in addition to paracrine expression of IGF-I within the kidney would have been reduced. In this study, circulating concentrations of IGF-I increased, but paracrine production may have fallen secondary to suppression of endogenous growth hormone secretion. Alternatively, the effects of suppressing growth hormone secretion on chronically increased renal expression of IGF-I and its binding proteins and subsequent effects on renal haemodynamics may take longer than seven

days to manifest following commencement of rhIGF-I/rhIGFBP-3 complex therapy or a higher dose may be needed. ACE inhibitors have effects on GFR within 24 hours in young people with Type 1 Diabetes (Hollenberg, Price et al. 2003). In addition IGF-I and growth hormone have effects on GFR that manifest within 6 hours and 3 days respectively (Hirschberg and Kopple 1989; Hirschberg, Brunori et al. 1993). It is therefore reasonable to anticipate that any effect on GFR would be manifest after 7 days' treatment with rhIGF-I/rhIGFBP-3 complex.

There are several differences between this study and the murine study referred to above and the negative result of this study may reflect differences in the expression of growth hormone dependent peptides between mouse and man. There are little or no data in humans pertaining to expression of IGF-I and its receptors within the healthy human kidney or those of individuals with Type 1 Diabetes. Several studies report a direct relationship between urinary excretion of IGF-I and AER; but should be interpreted with caution in the light of a kidney that is leaking albumin, a much larger protein than IGF-I (Quattrin, Albini et al. 1992; Fujihara, Uemasu et al. 1996; Gill, Toogood et al. 1998). Nevertheless, human subjects with MA have higher urinary IGF-I than controls without MA, despite having lower concentrations of free IGF-I (Cummings, Sochett et al. 1998). However, concentrations of peptide hormones in urine depend not only upon local renal concentrations but also on tubular function (Hourd, Edge et al. 1991). It is unclear whether urinary IGF-I excretion mirrors renal paracrine expression of IGF-I, or represents plasma IGF-I that has been filtered and excreted.

Following treatment with rhIGF-I/rhIGFBP-3 complex, but not placebo, there was an increase in the urinary excretion of IGFBP-3 but not IGF-I, the significance of which is not clear from this work. In the absence of increase in the excretion of any other markers such as RBP or albumin, it appears to be specific for IGFBP-3. In the following chapter, an apparent induction of IGFBP-3 proteolysis following administration of rhIGF-I/rhIGFBP-3 complex is reported which may shed some light on this finding. In general,



following administration of rhIGF-I/rhIGFBP-3 complex, the detectable rise in plasma IGFBP-3 concentrations is proportionately smaller than that seen in concentrations of IGF-I. If there is an induction of proteolysis, with subsequent excretion of smaller fragments of IGFBP-3 in the urine, that may go some way to explaining the discrepancy.

The small sample size of only 6 subjects is a limitation of this work and a larger sample size may have revealed an effect. However, we may exclude a large effect of rhIGF-I/rhIGFBP-3 complex on GFR (a change in mean GFR of 30 ml/min/1.73m<sup>2</sup>), which would have returned our hyperfiltrating subjects to the reference range for GFR.

Following rhIGF-I/rhIGFBP-3 complex treatment; all subjects remained within the hyperfiltrating range. A larger sample size would detect smaller effects on GFR, but may not have been clinically relevant.

Finally, other potential confounders cannot be discounted. Treatment with rhIGF-I/rhIGFBP-3 complex for 7 days led to reductions both in insulin requirements and plasma insulin concentrations in the 5 hours prior to determination of GFR. Increasing insulin concentrations lead to an increase in renal retention of sodium and water and an increase in GFR (ter Maaten, Voordouw et al. 1999). We therefore might have expected GFR to decrease secondary to reductions in overnight plasma insulin concentrations following treatment with rhIGF-I/rhIGFBP-3 complex.

### 3.5. Conclusions

In conclusion, seven days adjunctive therapy with rhIGF-I/rhIGFBP-3 complex (dose 0.4mg/kg/day equivalent to 80mcg/kg/day IGF-I) was well tolerated and appeared safe. We observed an increase in overnight insulin sensitivity and a reduction in creatinine concentration, coupled with a reduction in overnight growth hormone secretion, but did not observe any changes either in glomerular filtration or urinary albumin excretion rates. Further work, detailing the effects of longer term adjunctive therapy with rhIGF-I/rhIGFBP-3 complex in subjects with microalbuminuria on urinary albumin excretion rates would be of interest, along with larger scale clinical trials of rhIGF-I/rhIGFBP-3 in



the Type 1 Diabetes population, considering perhaps more clinically relevant outcomes such as HbA1c, rates of hypoglycaemia and total daily insulin dose along with side effect profile would be of interest. It would also be of interest to examine the effects of the growth hormone antagonist, B2036-PEG on GFR and urinary albumin excretion rates as this should in theory reduce both circulating concentrations of IGF-I in addition to paracrine IGF-I within the kidney in a fashion analogous to that described in the mouse model by Flyvbjerg and colleagues.

## **Chapter 4: THE EFFECTS OF rhIGF-I/rhIGFBP-3 COMPLEX ON IGFBP-3 PROTEOLYSIS IN Type 1 Diabetes**

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### **4.1. Introduction**

The laboratory work described in this chapter was performed on samples collected during a study co-ordinated by Dr Tero Saukkonen which explored the effects of rhIGF-I/rhIGFBP-3 complex administration (Mecasermin rinfabate, Somatokine, iPLEX™) on insulin sensitivity in young people with type one diabetes. The study was a dose-ranging study (0.1 to 0.8mg/kg/day), looking at the effects of increasing doses of rhIGF-I/rhIGFBP-3 complex on overnight insulin requirements and insulin sensitivity during a hyperinsulinaemic clamp the following day and demonstrated a dose dependent increase in overnight insulin sensitivity in addition to increased peripheral insulin sensitivity during the hyperinsulinaemic clamp (Saukkonen, Amin et al. 2004; Saukkonen, Shojaei-Moradie et al. 2006). During the analysis of the samples, it was noted that although IGF-I concentrations rose in a dose-dependent fashion as expected following the administration of rhIGF-I/rhIGFBP-3 complex, the rise in IGFBP-3 concentrations was less marked, despite the fact that rhIGF-I/rhIGFBP-3 complex consists of IGF-I and IGFBP-3 in equimolar proportions. In conjunction with the increases in urinary excretion of IGFBP-3 demonstrated in the previous chapter, this prompted us to look more closely at any effects of rhIGF-I/rhIGFBP-3 complex on proteolysis of IGFBP-3. Western immunoblotting analysis was used in order to investigate any potential effects of rhIGF-I/rhIGFBP-3 complex on IGFBP-3 proteolysis and modified immunobased assays were used to quantify urinary concentrations of IGFBP-3. The work described in this chapter was undertaken in the laboratory of Dr. Ceci Camacho-Hübner, at St Bartholomew's Medical School, London, UK and I am grateful to her and particularly to Dr Farideh Miraki-Moud for their guidance and patience.

## 4.2. Methods

### 4.2.1 *Study Design*

The study was a double blind, randomised crossover study of low (0.1 and 0.2 mg/kg) and high (0.4 and 0.8mg/kg) dose rhIGF-I/rhIGFBP-3 complex in young people with type one diabetes versus placebo. All subjects were studied on 3 separate occasions; receiving placebo on one occasion and rhIGF-I/rhIGFBP-3 complex on the other 2. Subjects were randomised into 2 groups; group A received placebo, rhIGF-I/rhIGFBP-3 complex 0.1mg/kg and rhIGF-I/rhIGFBP-3 complex 0.4mg/kg in random order and group B received placebo, rhIGF-I/rhIGFBP-3 complex 0.2mg/kg and rhIGF-I/rhIGFBP-3 complex 0.8mg/kg on 3 separate occasions in random order. There was a minimum washout period of 4 weeks between each study.

### 4.2.2 *Subjects*

8 young adult subjects with Type 1 Diabetes were recruited for the study (5M, age 13.6-18.8y, mean HbA1c 9.9% (range 8.0 to 11.9)). All were post pubertal, with Type 1 Diabetes of at least 1years' duration, and were C-peptide negative. All had normal renal, hepatic and thyroid function and a normal full blood count. None had any evidence of microalbuminuria or retinopathy. All were treated with a combination of short and long acting insulins two to four times per day (mean insulin dose 1.0 (0.7 to 1.4) units/kg/24hr). An additional single subject (subject 9) (male, aged 35y) agreed to participate in a modification of the study protocol following ethical approval by the Cambridge Local Regional Ethics Committee.

### 4.2.3 *Study Protocol*

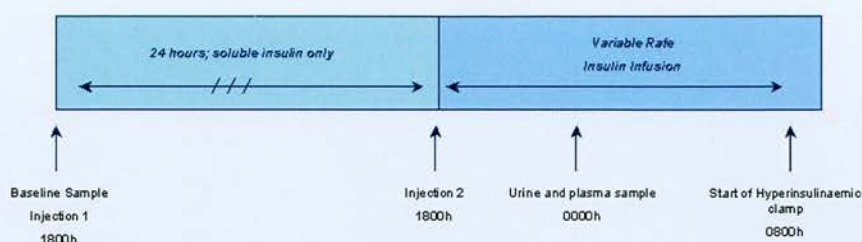
Subjects were studied on 3 separate occasions. On each occasion, subjects received 2 subcutaneous injections of either placebo or rhIGF-I/rhIGFBP-3 complex (0.1, 0.2, 0.4 or 0.8mg/kg), 24 hours apart at 1800h. rhIGF-I/rhIGFBP-3 complex was injected by the subjects under supervision into the anterolateral aspect of the left thigh. All medium and

long acting insulins were withdrawn 24 hours prior to the first injection and subjects maintained their blood glucose by using frequent injections of soluble insulin.

Following the second injection, subjects underwent an overnight variable rate insulin infusion for euglycaemia as described in chapter 2. Plasma samples (Lithium heparin) taken at baseline (prior to the first injection) and at midnight during the overnight samples were subsequently analysed for total IGF-I and IGFBP-3 using standard ELISA techniques and evaluation of IGFBP-3 proteolysis using Western immunoblotting.

A urine sample was collected at midnight on the second night for quantification of urinary IGFBP-3. Investigation of the forms of IGFBP-3 excreted in the urine was determined using Western immunoblotting techniques. Timing of injections and sampling is shown schematically in Figure 21.

An additional subject (subject 9, male, aged 35y) underwent a modified sampling protocol as follows. He was admitted to the WTCRF at 1700h on day 1 and an intravenous cannula was inserted for the purposes of sampling, and a baseline sample was taken at 1730h. At 1800h he received an injection of rhIGF-I/rhIGFBP-3 complex (0.4mg/kg). Thereafter, samples were taken hourly until 0600h the following morning. The following day, he returned to the WTCRF. A baseline sample was again taken at 1730h before he received his second injection at 1800h. Hourly samples were then taken until 0600h the following morning when he was discharged. He did not undergo overnight insulin infusion for euglycaemia or a hyperinsulinaemic clamp. Successive hourly samples were subjected to Western immunoblotting, IGF-I, IGFBP-3 and quantification of functional IGFBP-3.



**Figure 21: Study Schedule**

Diagrammatic representation of the study schedule. Timing of injections and sampling on each occasion are marked with arrows

#### 4.2.4 Urine Sample Collection, Preparation and Storage

Urine samples were collected at midnight on the second night, during the overnight period of euglycaemia, 30 hours after the first injection. 20ml samples were collected into plain falcon tubes and spun at 3000 rpm for 5min to precipitate any debris. 1.5ml aliquots were then stored at -80°C until analysis.

For Western Immunoblotting and quantification of IGFBP-3, urine samples were concentrated 10-fold prior to analysis. Samples were defrosted at room temperature and then concentrated 10-fold at 1500 rpm at 4°C (microfuge filters NMWL 10,000, Sigma, Dorset, UK). 1600µl of urine was concentrated to 160µl in a microfuge, 10000rpm in 400µ aliquots. The final volume was determined accurately using a Gilson pipette.

For determination of creatinine, samples were not concentrated prior to analysis.

#### 4.2.5 Laboratory Methods

##### 4.2.5.i Quantitative assay techniques: plasma

Plasma (total) IGF-I concentrations were measured in ethanolic extracts by ELISA, (DSL, Tooting, London, UK). The sensitivity of this assay was 0.03 ng/mL. Intra-assay imprecision (CV) was 8.8% and 9.4% at 107 and 262ng/ml respectively.



Plasma IGFBP-3 levels were measured by ELISA, (DSL, Tooting, London). The sensitivity of this assay was 0.04ng/mL. Intra-assay imprecision (CV) was 4.9% and 2.8% at 5.2 and 34.7ng/ml respectively. Equivalent inter-assay imprecision (CV) was 9.7% and 1.9% at 5.2 and 34.7ng/ml respectively.

#### 4.2.5.ii Quantitative Assay Techniques: Urine

All plasma and urine samples were stored at -80°C prior to analysis. For Western Immunoblotting and quantification of IGFBP-3, urine samples were concentrated 10-fold prior to analysis. Samples were defrosted at room temperature and then concentrated 10-fold at 100g and 4 °C (microfuge filters NMWL 10,000, Sigma, Poole, Dorset, UK). Urinary concentrations of IGFBP-3 were determined using a commercially available polyclonal ELISA kit (DSL, Tooting, London, UK). Prior to analysis urine was concentrated 10 fold (Centricon). The standard method was adapted to incorporate incubation of samples with the primary antibody for 18 hours at 4°C.

#### 4.2.5.iii Western Immunoblotting

Details of the reagents and methods for Western Immunoblotting are in Appendix 4.

#### 4.2.5.iv Scanning densitometry

Scanning densitometry analysis of the autoradiographs was performed. Firstly, a BioRad GS-670 scanning densitometer (Global Medical Instrumentation Inc, Ramsey, Minnesota, USA) was used to scan the autoradiographs and subsequently Molecular Analyst PDQuest densitometry software was used to quantify bands having subtracted background (Bio-Rad, Hercules, CA, USA ).

#### 4.2.6 *Calculations*

Following scanning densitometry of autoradiographs of western immunoblots, the relative density of each band, including intact IGFBP-3 and any fragments was expressed as a percentage of the total of the bands in each lane, after having been corrected for background.



#### 4.2.7 *Statistical Analysis*

For the purposes of analysis, data obtained from rhIGF-I/rhIGFBP-3 complex 100 and 200 µg/kg and rhIGF-I/rhIGFBP-3 complex 400 and 800 µg/kg were combined as low dose and high dose respectively in the 48 hour study.

Where data were normally distributed they were analysed using parametric tests.

Urinary concentrations of IGFBP-3 were not normally distributed and therefore were log transformed (base 10) to permit parametric testing. p values <0.05 (two tailed) were considered significant throughout.

All analyses were performed using SPSS for Windows, version 10. Student's t-tests for paired variables were used to compare within individual differences between plasma IGF-I and IGFBP-3 concentrations at time 0 and time +30h. A multivariate model incorporating subject ID and rhIGF-I/rhIGFBP-3 complex dose (low dose or high dose) as fixed factors was used to determine differences in urinary IGFBP-3 concentration between groups, with Dunnett's post hoc analysis of compared means used to determine differences between high dose and low dose groups.

Data are presented as mean  $\pm$  SEM or median (IQR) otherwise stated. p values of less than 0.05 (2-tailed) were considered to be statistically significant.

### 4.3. Results

Data are presented as mean  $\pm$  SEM unless otherwise stated.

#### 4.3.1 *Plasma Concentrations of IGF-I and IGFBP-3*

There was no difference in either IGF-I or IGFBP-3 concentrations at time 0 between placebo, low dose or high dose groups (Table 11). Plasma concentrations of total IGF-I were higher at time +30h than at time 0 with high dose rhIGF-I/rhIGFBP-3 complex, but not with placebo or with low dose rhIGF-I/rhIGFBP-3 complex (Table 11). Plasma concentration of IGFBP-3 did not change between time 0 and time +30h either with low dose or high dose rhIGF-I/rhIGFBP-3 complex treatment (Table 11). The IGF-I/IGFBP-

3 molar ratio was increased following treatment with high dose rhIGF-I/rhIGFBP-3 complex (Table 11).

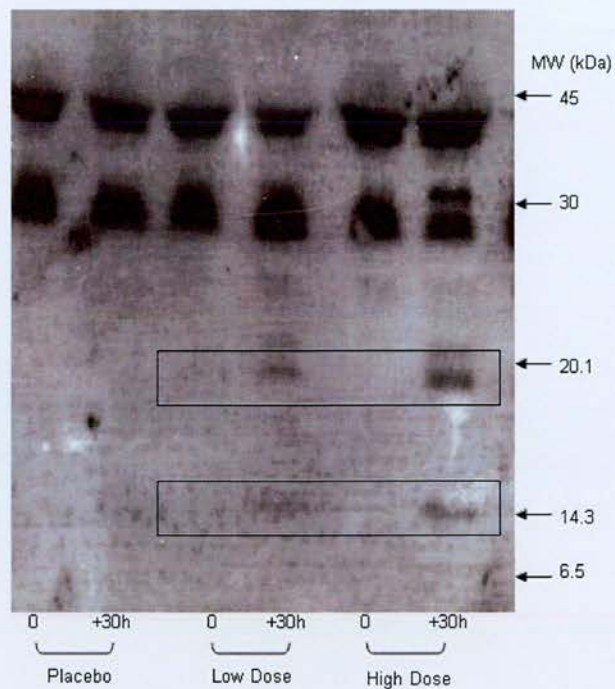
	Plasma IGF-I (ng/ml)		Plasma IGFBP-3 (mg/l)		IGF-I/IGFBP-3 molar ratio		Urinary IGFBP-3 (mcg/mmol creatinine)
	Time 0	+30h	Time 0	+30h	Time 0	+30h	+30h
Placebo	211 ± 29	157 ± 26	4.9 ± 0.3	4.3 ± 0.2	0.24 ± 0.04	0.20 ± 0.03	59 ± 9
Low Dose	201 ± 44	235 ± 53	4.7 ± 0.2	4.5 ± 0.2	0.23 ± 0.05	0.28 ± 0.04	127 ± 34 †
High Dose	219 ± 33	458 ± 70 **	5.1 ± 0.3	5.0 ± 0.2	0.23 ± 0.04	0.48 ± 0.07 *	177 ± 18 ††

Table 11. IGF-I and IGFBP-3 Concentrations in plasma and urine

Plasma and urinary IGF-I and IGBP-3 concentrations before and after placebo and rhIGF-I/rhIGFBP-3 complex. \* p<0.05 vs pre, \*\* p<0.005 vs pre, † p<0.05 vs placebo, †† p<0.001 vs placebo.

#### 4.3.2 IGFBP-3 Proteolysis

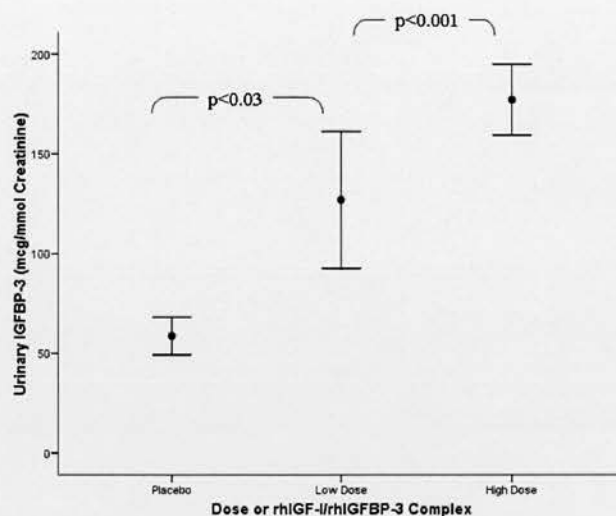
Western Immunoblotting of serum samples at time 0 revealed the expected immunoreactive bands at 45 and 30 kDa (Figure 22). Following rhIGF-I/rhIGFBP-3 complex treatment, at time +30h, 2 additional immunoreactive bands of approximate sizes 18- and 14- kDa were present which were not there at time 0 in any subject (Figure 22). The fragments were present in 7 out of 8 subjects in the low dose group and in all subjects in the high dose group.



**Figure 22: Western Immunoblot of Serum Samples** Representative Western Immunoblot of plasma before (time 0) and after (time +30h) placebo, low dose and high dose rhIGF-I/rhIGFBP-3 complex. Fragments at 14- and 18- kDa following treatment with rhIGF-I/rhIGFBP-3 complex are shown within the box. *Urinary Excretion of IGFBP-*

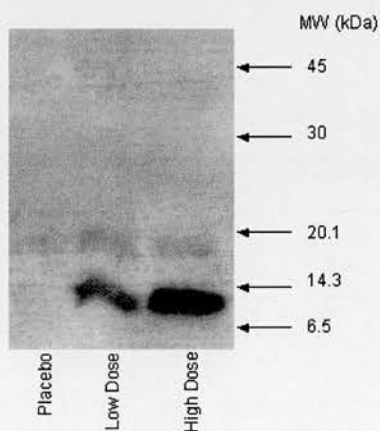
3

Urinary excretion of IGFBP-3 at time +30h increased in a dose-dependent fashion following rhIGF-I/rhIGFBP-3 complex treatment;  $58.8 \pm 9.4$   $\mu\text{g}/\text{mmol}$  creatinine following placebo vs  $127.0 \pm 34.3$  following low dose rhIGF-I/rhIGFBP-3 complex and  $177.2 \pm 17.8$  following high dose rhIGF-I/rhIGFBP-3 complex (Table 11, Figure 23).



**Figure 23: Urinary Excretion of IGFBP-3**

Effects of low dose (0.1 and 0.2mg/kg) and high dose (0.4 and 0.8 mg/kg) rhIGF-I/rhIGFBP-3 complex on urinary excretion of IGFBP-3 (ELISA). Differences against placebo explored using a univariate model with urinary BP-3 as the dependent variable, incorporating subject ID and treatment group as fixed factors. Error Bars represent 1 SEM.



**Figure 24: Western Immunoblot of Urine Samples**

Immunoblot of urine samples +30h following injection of Placebo, low dose and high dose rhIGF-I/rhIGFBP-3 complex. Fragments of approximate molecular weight 14- and 18-kDa are present following rhIGF-I/rhIGFBP-3 complex, but not placebo.

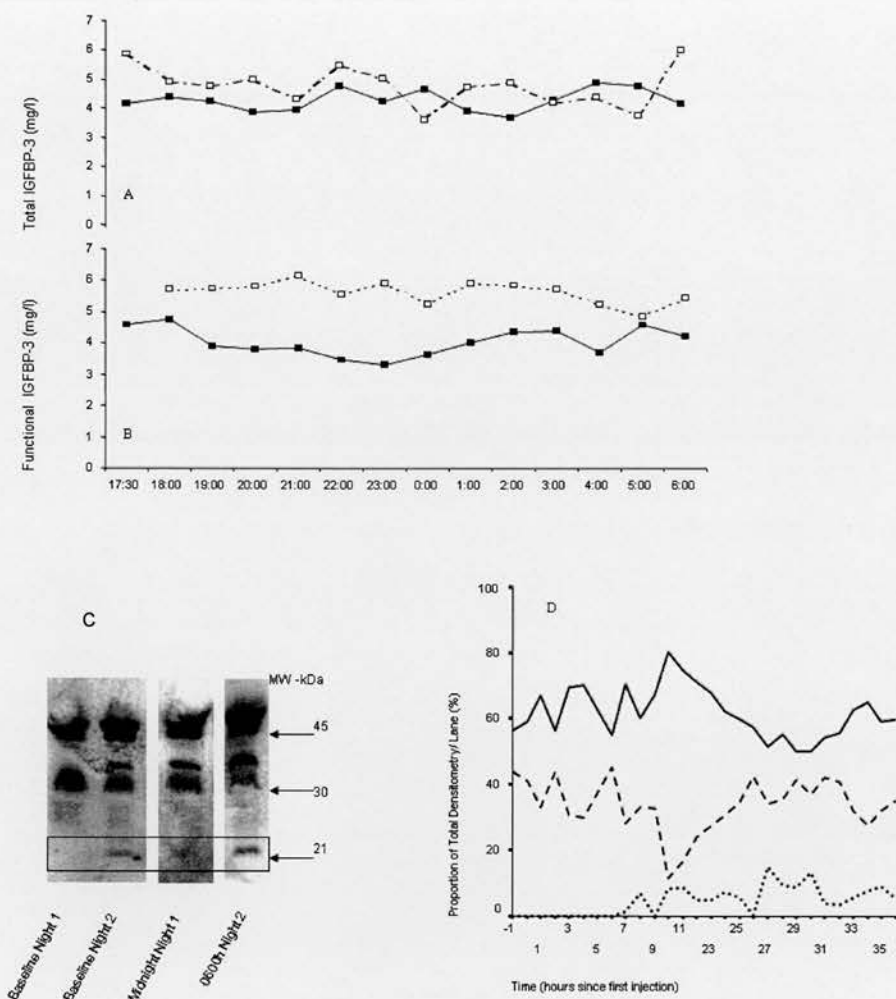
#### 4.3.4 *Urinary IGFBP-3 Fragments*

Western Immunoblotting for IGFBP-3 of urine samples taken at time +30h revealed 2 immunoreactive bands of approximate molecular weights 14- and 18-kDa (Figure 24). Intact IGFBP-3 was not seen.

#### 4.3.5 *Timing of Appearance of IGFBP-3 Fragments*

Subject 9 underwent a modified sampling protocol as described. Briefly, he received 2 injections of rhIGF-I/rhIGFBP-3 complex; 0.4mg/kg 24 hours apart, administered at 1800h as did the other subjects, but he had samples taken hourly overnight following the first injection, and then again following the second. Blood samples were analysed for IGF-I concentration and total and functional IGFBP-3 concentrations. Western Immunoblotting followed by densitometry was then performed on all samples in order to determine the timing of appearance of the IGFBP-3 fragments.

With only data from one subject, it was not appropriate to perform statistical comparison between the 2 nights. However, although there was no apparent difference in the concentrations of total IGFBP-3 between the 2 nights, it appeared that the amount of functional IGFBP-3 (that able to bind ligand) was increased on the second of the sampling nights (Figure 25, panels A and B). Similar effects on proteolysis were noted in subject 9 following rhIGF-I/rhIGFBP-3 complex administration as had been observed in the previous subjects, with appearance of a fragment of approximately 18 –kDa which was first detectable approximately 7 hours after the first dose of rhIGF-I/rhIGFBP-3 complex (Figure 25, panels C and D).



**Figure 25: Results from sequential samples in subject 9**

A: Total IGFBP-3 as determined by ELISA. Night 1 solid line and filled squares, night 2 broken line and open squares. B: Functional IGFBP-3. Night 1 solid line and filled squares, night 2 broken line and open squares. C: Representative immunoblotting showing the appearance of 18-kDa fragment (within box) 7 hours after the initial injection and its persistence through to the end of sampling in addition to the immunoreactive bands at 45 and 30 -kDa. D: Densitometry following western immunoblotting from all sequential samples. Solid line represents 45-kDa IGFBP-3, Broken line 30 -kDa, Dotted line, fragments.



#### 4.4. Discussion

The data reported here indicate that, following the administration of rhIGF-I/rhIGFBP-3 complex to young adults with Type 1 Diabetes, there is an induction of the proteolysis of IGFBP-3, leading to the generation of fragments which are excreted in the urine in a dose-dependent fashion. The data reported in Chapter 3 suggested that although there is an increase in the urinary excretion of IGFBP-3 following treatment with rhIGF-I/rhIGFBP-3 complex, the urinary excretion of IGF-I (the other component of the complex) remains unchanged, as does the excretion of retinol binding protein, a non-specific marker of tubular dysfunction and albumin. This implies that the excretion of fragmented IGFBP-3 is a specific process, rather than secondary to a general increase in renal tubular proteinuria following administration of rhIGF-I/rhIGFBP-3 complex.

The administration of IGF-I complexed to its natural carrier protein; IGFBP-3 has been explored as a way of exploiting the insulin sensitising effects of IGF-I, whilst prolonging its half-life within the circulation and potentially minimising side effects. To date, studies of rhIGF-I/rhIGFBP-3 complex administration to adults with both type 1 and type 2 diabetes, adolescents with Type 1 Diabetes and a small group of subjects with growth hormone insensitivity syndrome (GHIS) have been reported (Clemmons, Moses et al. 2000; Clemmons, Moses et al. 2005; Clemmons, Sleevi et al. 2005; Camacho-Hubner, Rose et al. 2006).

In this chapter, short-term administration of rhIGF-I/rhIGFBP-3 complex to young adults with Type 1 Diabetes resulted in a dose dependent increase in plasma concentrations (approximately four fold in the high dose group) whereas the relative molar increase in IGFBP-3 concentrations was much lower. These increases are similar to those reported with the administration of rhIGF-I/rhIGFBP-3 complex to adults with T2D where a 4 to 5 fold increase in total IGF-I concentrations compared with a 1.6 fold increase in IGFBP-3 concentrations was observed (Clemmons, Moses et al. 2005). In adults with Type 1 Diabetes a similar, four fold increase in IGF-I was observed,

although changes in IGFBP-3 were not reported (Clemmons, Moses et al. 2000). In another study of four subjects with growth hormone insensitivity syndrome (GHIS), administration of the rhIGF-I/rhIGFBP-3 complex lead to four fold molar increases in IGF-I concentrations, but only a 1.4 fold increase in IGFBP-3 (Camacho-Hubner, Rose et al. 2006). Western blotting revealed the appearance of a 30 -kDa band following administration of the complex, but the authors did not comment on its likely significance. The data reported here are similar, with IGF-I concentrations increased four fold, with a much smaller (around 15%) increase in IGFBP-3 concentrations.

Additional data from an additional single subject who underwent hourly sampling from administration of the first dose of rhIGF-I/rhIGFBP-3 complex indicates that there may be an increase in 'functional' IGFBP-3, i.e. that which is capable of binding ligand. Clemmons and co-workers have reported that following administration of rhIGF-I/rhIGFBP-3 complex to adults with diabetes, there is an induction of IGFBP-3 proteolysis which results in preferential degradation of the non-glycosylated form of the protein (i.e. the exogenously administered form), with the glycosylated form being protected (Clemmons, Sleevi et al. 2005). This may lead to an overall increase in glycosylated IGFBP-3 available for ligand binding, which may in turn result in a more stable pool of IGF-I within the circulation. However, far more detailed studies involving larger numbers are required before the exact nature of the effect of administration of rhIGF-I/IGFBP-3 complex on IGF binding proteins, the ternary complex and IGF-I bioavailability can be determined.

There are several potential explanations for the apparent lack of increase in plasma IGFBP-3 concentrations following rhIGF-I/rhIGFBP-3 complex. One possibility is that the plasma concentrations *are* increased but that it could not be detected using the quantification method. However, the assay used, a commercially available ELISA incorporates a polyclonal antibody that should bind both intact IGFBP-3 in addition to fragments. Similarly, polyclonal antibodies were used in the Western immunoblot and

therefore should have detected all forms of IGFBP-3. As the same antibodies were able to detect increasing amounts of immunoreactive IGFBP-3 in urine, it would appear that the relatively smaller rise in IGFBP-3 concentrations following rhIGF-I/rhIGFBP-3 complex administration reflects the urinary excretion of fragments rather than the failure to detect fragments in plasma by the assay.

Alternatively, IGF-I from the complex may be preferentially absorbed over IGFBP-3, which might not enter the circulation. However, in the preclinical development phase of rhIGF-I/rhIGFBP-3 complex, it was reported that in rats and monkeys that administration resulted in binding of the complex to endogenous ALS within the circulation, indicating that, at least in animals, the complex is intact (Adams, Moore et al. 1995). The data from subject 9 demonstrate the first appearance of plasma fragments 7 hours after the first injection, indicating that the induction of proteolysis is a relatively acute phenomenon.

Finally, IGFBP-3 may be degraded following absorption and subsequently cleared from the circulation. The most plausible explanation for this would be via induction of intravascular proteolysis with renal clearance of generated fragments. The data presented in this chapter provide support for this, with demonstration of the appearance of IGFBP-3 fragments in serum following the administration of rhIGF-I/rhIGFBP-3 complex, with fragments of similar molecular weight detectable in the urine in a dose-dependent fashion. The data presented here do not add any additional information regarding the site of proteolysis, which may have occurred prior to absorption from the injection site, within the circulation, or within the tissues, for example, the kidney prior to excretion.

Urinary excretion of an N-terminal fragment of IGFBP-3 of approximately 18- kDa has been reported to be greater in children than adults and reduced in children with GHD, increasing following treatment with growth hormone, implying growth hormone dependency (Spagnoli, Gargosky et al. 1995). In Chapter 3, it was demonstrated that administration of rhIGF-I/rhIGFBP-3 complex to young people with Type 1 Diabetes

results in suppression of endogenous growth hormone secretion and therefore a reduction rather than an increase in urinary IGFBP-3 might be anticipated. Finally, increased excretion of an 18 –kDa N terminal fragment of IGFBP-3 in conjunction with proteolysis has been described in adolescents with Type 1 Diabetes and microalbuminuria when compared to controls without microalbuminuria, with urinary concentration of the IGFBP-3 fragment correlating with proteolysis quantified by immunoblotting techniques (Spagnoli, Chiarelli et al. 1999). However, in that study, the urinary IGFBP-3 correlated strongly with albumin excretion rates which might reflect non specific tubular protein leak rather than a specific process. Alternatively it may be secondary to the increased growth hormone secretion which is reported in patients with Type 1 diabetes and microalbuminuria (Amin, Williams et al. 2005).

It is not clear why fragments are excreted, as peptides of this size would normally be reabsorbed by the renal tubules and recycled. The dose dependent increases in the urinary excretion of IGFBP-3 fragments following rhIGF-I/rhIGFBP-3 complex may simply represent a mechanism for excretion of excess, although it would be more likely that they be reabsorbed, degraded and recycled rather than simply being excreted.

Tubular re-absorption of peptides is an exquisitely ATP dependent process and all samples were taken following an overnight period of euglycaemia. Small urinary peptides such as retinol binding protein (RBP) and N-acetyl glucosaminidase (NAG) are present in up to 60 and 100% respectively of children with diabetes 5 years from diagnosis, but are not reliable indicators of subsequent microalbuminuria risk, although they are related to duration of diabetes and HbA1c (Schultz, Dalton et al. 2001) . This implies that the mechanisms for the tubular re-absorption of peptides in young people with Type 1 Diabetes are somehow impaired and may in part explain these findings.

IGFBP-3 proteolysis has been reported in children with Type 1 Diabetes prior to commencement of insulin and in diabetic ketoacidosis, i.e. it appears to be a marker for catabolism (Bereket, Lang et al. 1995). IGF-I has well recognised anabolic effects and it

is unlikely that our subjects were more catabolic following complex administration. Indeed, in chapter 3, following administration of rhIGF-I/rhIGFBP-3 complex there was a reduction in creatinine concentrations in the absence of any change in GFR, implying an increase in anabolism rather than catabolism (Williams, Yuen et al. 2006).

Clemmons and co-workers reported that following administration of rhIGF-I/rhIGFBP-3 complex to adults with T2D, there is induction of IGFBP-3 proteolysis apparently specific for the non-glycosylated form (Clemmons, Sleevi et al. 2005). The authors argued that by acting as a preferential substrate for protease activity, the non-glycosylated IGFBP-3 protects endogenous glycosylated IGFBP-3 from degradation, leading to increases in total IGFBP-3 proportionately less than the increases in IGF-I, but act to increase the half life of IGF-I by increasing stability (Clemmons, Sleevi et al. 2005).

A limitation of this study is the absence of any data from a healthy control population without diabetes. It is notable that, with the exception of the early human studies in the development of rhIGF-I/rhIGFBP-3 complex, none of the published work contains a control group for comparison, with almost all studies being of longitudinal design, studying individuals prior to and then following administration of rhIGF-I/rhIGFBP-3 complex. These studies, while they lack a control group, do report data from the same individuals following administration of placebo. In the subjects described here, there were no detectable fragments of IGFBP-3 in either plasma or urine in any subject either before or after administration of placebo, which has to be considered as robust control data and supports the argument that the fragments appear directly as a result of administration of rhIGF-I/rhIGFBP-3 complex to subjects with type 1 diabetes, although the precise mechanisms and significance remain unclear.

#### 4.5. Conclusion

Administration of an equimolar rhIGF-I/rhIGFBP-3 complex in people with Type 1 Diabetes leads to disproportionate increases in plasma IGF-I and IGFBP-3

concentrations. The demonstration of fragments within plasma and dose-dependent increase in urinary IGFBP-3 concentrations suggest that this may result from IGFBP-3 proteolysis and subsequent urinary excretion. It is possible that the induction of proteolysis of IGFBP-3 following administration of the recombinant complex may have implications for the bioefficacy of the compound.

These data suggest that administration of rhIGF-I/rhIGFBP-3 complex to young adults with Type 1 Diabetes results in an induction of the proteolysis of IGFBP-3 with subsequent excretion of fragments of IGFBP-3 in the urine. The significance of these findings is not clear but an increase in proteolytic activity may lead to an increase in bioavailable IGF-I and reflect a more subtle mechanism for regulation of availability of bioactive IGF-I.



## Chapter 5: Conclusions

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### 5.1. Introduction

The central vein of this thesis is the dysregulation of the growth hormone - IGF-I axis which is present in young adults with Type 1 Diabetes; namely, low circulating concentrations of IGF-I, with subsequent hypersecretion of growth hormone due to failure of the negative feedback mechanism. This stems from low concentrations of insulin within the portal circulation with subsequent reductions in expression of the hepatic growth hormone receptor and hepatic IGF-I generation. Currently, despite the development of novel insulin analogues and technology such as CSII and glucose sensors, the alternatives for insulin replacement in adolescents with Type 1 Diabetes remain far from physiological. The eventual development of closed loop systems, may achieve normoglycaemia for the majority of the time, but optimisation both of glucose sensing technology and algorithms has yet to be achieved (Hovorka 2006). Despite this, insulin administration will remain peripheral and, as such the dysregulation of the growth hormone - IGF-I axis will only be partially corrected (Amiel, Sherwin et al. 1984). Insulin delivery to the portal vein as part of a closed loop system is unlikely to be a viable alternative, although peritoneal delivery is being actively explored as an alternative (Gin, Renard et al. 2003).

### 5.2. The Strengths and Weaknesses of the Reported Studies

The work detailed in this thesis demonstrates the limitations of physiological studies in the human (or indeed in any other whole animal system); in that it is impossible to manipulate one component of a complex and finely regulated system without affecting others. Antagonism of the actions of growth hormone leads to reductions in circulating IGF-I concentrations and increasing IGF-I concentrations leads to suppression of growth hormone secretion and the more subtle effects, particularly at the cellular level can only be surmised. The beneficial effects of specific growth hormone blockade overnight,

apparently mediated by an effect on lipolysis, were not observed during the hyperinsulinaemic clamp which may be due to the inevitable consequence of growth hormone antagonism, reductions in IGF-I concentrations. Conversely, when IGF-I was replaced in the form of rhIGF-I/rhIGFBP-3 complex, as well as increases in IGF-I, we observed the anticipated reductions in endogenous growth hormone secretion. Growth hormone and IGF-I *both* lead to increases in glomerular filtration rate, so it might be that the effects of growth hormone suppression on GFR were counteracted by the opposing effects of IGF-I. In addition we had no information regarding paracrine effects of growth hormone on intrarenal IGF-I expression; to gather such information would require serial renal biopsies, and, while it would undoubtedly be of scientific interest, would be inappropriately invasive.

Some insight into the potential knock on effects on manipulation of the growth hormone - IGF-I axis was obtained from the work on IGFBP-3 proteolysis detailed in Chapter 4 although the exact significance of it remains unclear. Administration of IGF-I complexed with IGFBP-3 led to an apparent induction of IGFBP-3 proteolysis, which presumably reflects an effect on the subtle regulation of IGF-I bioavailability although the significances of this remain unclear. The excretion of fragments of IGFBP-3 in the absence of any evidence of a non specific increase in tubular protein loss, and in the absence of an increase in urinary IGF-I concentrations is extremely interesting as in theory the fragments should be reabsorbed and their amino acids recycled. We were not able to determine whether the excreted fragments were filtered and not reabsorbed or whether they were actively excreted by the renal tubules. The significance of these observations is hard to interpret in the context of these studies but may have implications for the bio-efficacy of rhIGF-I/rhIGFBP-3 complex.

The use of animal models and cell biology allow more detailed and invasive study of biological parameters, however the findings have to be interpreted with caution when considering the human. In particular, while a great deal of detail can be gleaned from

animal models such as knock out mice, the effects of a gene knockout from birth in a different species will be different to the situation of a human who develops autoimmune mediated diabetes in childhood and who has previously had normal physiological regulation of growth hormone and its dependent peptides. Cell biological techniques, while giving some insight into the intracellular pathways, have yet to reveal the full extent of the subtle and dynamic interplay between the different signalling pathways.

The information from all sources should be taken and interpreted appropriately.

Ultimately, however, if avenues such as manipulation of the growth hormone IGF-I axis in Type 1 Diabetes are to be explored, either as a tool to further our understanding of physiology, or as a potential therapeutic intervention, the biological effects, along with safety profiling have to be understood in the applicable situation, namely the human.

### 5.3. Limitations of the Methodology

The methods used in this work to explore human physiology can broadly be divided into 2: those attempting to model normative physiology as far as possible; for example the overnight studies which detail the overnight requirements for euglycaemia and the hyperinsulinaemic clamp methodology which uses an extreme in order to maximally stimulate glucose utilisation. It could be argued that the former are both more clinically and physiologically relevant in the adolescent with Type 1 Diabetes. Particularly in the work on selective growth hormone blockade, where the effects appeared to be mediated via growth hormone's effects on lipolysis, the overnight studies revealed more than the hyperinsulinaemic clamp where even the low dose of insulin used in the first step was sufficient to completely suppress lipolysis, thereby ameliorating any discernable effects on hepatic insulin sensitivity. Although the hyperinsulinaemic clamp is without doubt the gold standard method for assessing insulin sensitivity, it should be remembered that it was developed for the investigation of individuals with insulin resistance and type 2 diabetes and as such may not be the most useful tool for use in young adults with type 1 diabetes, particularly when exploring effects which are likely to be related to effects on

systems such as lipolysis which are exquisitely sensitive to insulin. Alternative methods such as utilising cold isotope modelling techniques during steady state euglycaemia during the overnight period may be a more useful tool.

#### 5.4. Adjunctive therapy in Type 1 Diabetes

The work presented in this thesis represents detailed physiological studies in small numbers of adolescents and young adults with Type 1 Diabetes. To date there have been no further studies of specific growth hormone blockade in either type 1 or type 2 diabetes published in the scientific literature. Two further avenues would be of interest in the young adult diabetic population with this approach. Firstly, the study of renal physiology and urinary albumin excretion rates following administration of a growth hormone antagonist to humans would be of interest, especially in the light of the extremely encouraging animal data. Secondly, given the reductions in overnight insulin requirements following specific growth hormone blockade, larger scale clinical trials to look in detail at clinical efficacy as well as to investigate long term safety data would be of interest as if increasing insulin requirements towards the dawn were ameliorated by a growth hormone antagonist. As such, achieving fasting blood glucose concentrations within target without concurrent nocturnal hypoglycaemia may be more achievable, however, the concomitant effects of further reductions in IGF-I would have to be considered. It would be interesting to compare both doses (5 and 10mg) as we observed reductions in overnight insulin requirements with the 5mg dose without significant reductions in IGF-I.

With respect to IGF-I, given either as rhIGF-I alone, or complexed to IGFBP-3, there is now a reasonably extensive literature pertaining to its use in both type 1 and type 2 diabetes, incorporating both physiological studies and longer term clinical trials. In general, adjunctive therapy with rhIGF-I led to reductions in insulin requirements and in 1 long term clinical trial in adolescents with Type 1 Diabetes, there were reductions in HbA1c over 3 months which were unfortunately not sustained over 6 months. This may

have been related to concordance issues and it must be acknowledged that enthusiasm for any additional therapy to insulin in young adults with Type 1 Diabetes which requires another injection on a day to day basis is likely to wane with time. The potential development of rhIGF-I was abruptly terminated in the late 1990s due to concerns regarding a high incidence of worrying side effects observed in patients using high doses. rhIGF-I/rhIGFBP-3 complex was developed as an alternative in the hope that the complex would be more stable and confer a more pharmacokinetically favourable profile with fewer dose related side effects. It has yet to be adequately established whether this is the case and rhIGF-I/rhIGFBP-3 complex is no longer being pursued as a therapy in the spectrum of insulin resistance.

### 5.5. Final Conclusions

The work towards this thesis has furthered understanding of the physiology of the growth hormone - IGF-I axis in adolescents with diabetes. Ultimately in the human model things can not be described in exquisite detail but nonetheless the importance of such studies should not be underestimated. Manipulation of parameters of the growth hormone - IGF-I axis in an attempt to restore more normative physiology, either by the suppression of growth hormone secretion or by the pharmacological blockade of its actions have been described. The studies incorporate detailed physiological methodology to quantify parameters of insulin sensitivity, carbohydrate and fat metabolism and renal physiology and are therefore expensive and time consuming to perform in only small numbers of subjects. Such studies provide valuable insight into the roles of different components of growth hormone and its dependent peptides in the *in vivo* human model and it is vitally important to explore in detail the physiological effects of such interventions in detail prior to any larger scale trials of clinical efficacy in order to fully detail their effects.



## Appendices

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### Appendix 1: Titration of Insulin Dose During Growth Hormone Antagonist Study

Active dose titration should always seek to achieve the best glycaemic control.

#### A1.1 Run In Phase

Insulin dose should be titrated according to blood glucose values from home blood glucose monitoring.

##### *A1.1.1 Long Acting:*

Intermediate acting (NPH / insulatard) should be titrated according to fasting / pre breakfast blood glucose (target range 5 to 8 mmol/l) in those on multiple injection therapy (MIT) and additionally to pre-dinner values in those on tds regimens (target range 5-8 mmol/l).

The NPH dose should be lowered if blood glucose values fall below the target range for 3 consecutive days or if there are episodes of symptomatic hypoglycaemia. Decrease the dose of NPH by at least 10% but not more than 20%.

The NPH dose should be increased if blood glucose levels are higher than target range for at least three consecutive days. Increase the dose of NPH by at least 10% but not more than 20%.

##### *A1.1.2 Short Acting:*

Soluble insulin should be injected 20 to 30 minutes before a meal and rapid acting insulin analogues should be injected 5 minutes before meals.

The titration of short/rapid acting insulin should be according to pre-lunch, pre-dinner and pre-bed blood glucose values (pre-dinner and pre-bed in tds regimens). The pre-lunch / dinner value should be between 5-8 mmol/L and the pre-bed value should be between 8-12mmol/l. If blood glucose is higher than target for more than 3 days, then increase short acting insulin by 10% of the dose. If blood glucose is lower than target for



3 consecutive days or in the event of symptomatic episodes of hypoglycaemia, short acting insulin should be decreased by 10% of dose.

#### A1.2 During Treatment

Those subjects taking rhIGF-I/IGFP-3 are likely to need to reduce their insulin dose by 25 to 30%. All subjects should reduce their insulin by 20% (both long and short acting components) following their second injection.

Thereafter, titrate insulin dose according to blood glucose values.

##### *A1.2.1 Long Acting:*

Intermediate acting (NPH / insulatard) should be titrated according to fasting / pre breakfast blood glucose (target range 5 to 8 mmol/l) in those on multiple injection therapy (MIT) and additionally to pre-dinner values in those on tds regimens (target range 5-8 mmol/l).

The NPH dose should be lowered if blood glucose values are below the target range for one day or if there are episodes of symptomatic hypoglycaemia. Decrease the dose of NPH by at least 10% but not more than 20%.

The NPH dose should be increased if blood glucose levels are higher than target range for at least two consecutive days. Increase the dose of NPH by at least 10% but not more than 20%.

##### *A1.2.2 Short Acting:*

Soluble insulin should be injected 20 to 30 minutes before a meal and rapid acting insulin analogues should be injected 5 minutes before meals.

The titration of short/rapid acting insulin should be according to pre-lunch, pre-dinner and pre-bed blood glucose values (pre-dinner and pre-bed in tds regimens). The pre-lunch / dinner value should be between 5-8 mmol/L and the pre-bed value should be between 8-12mmol/l. If blood glucose is lower than target for 1 day, or in the event of

symptomatic episodes of hypoglycaemia, short acting insulin should be decreased by 10% of dose. If blood glucose is higher than target for more than 3 days, then increase short acting insulin by 10% of the dose.

### A1.3 Soluble Insulin Dose Prior to Overnight Euglycaemic Clamp

Long acting insulin was omitted for the 36 hrs prior to admission for the overnight insulin infusion for euglycaemia. During this period, subjects liaised closely with a member of the team who was not directly involved in the overnight insulin infusion (Dr Kevin Yuen). Soluble insulin (actrapid) was used in place of evening long acting by giving approximately 30% of the usual dose of isophane, or 50% of the usual dose of glargine. Thereafter, actrapid was given with meals according to blood glucose. If the blood glucose was above 10mmol/l pre meal, the subject's usual dose was increased by 10 to 15%. There were no problems with hypoglycaemia or ketotic hyperglycaemia in any subject during the 36hrs prior to admission following withdrawal of basal insulin.

## Appendix 2: Reference Chart for overnight sampling

Clock Time	Sample No.	Vol ml	Blood glucose	Growth Hormone	Free Insulin	NEFA	ACA	B-OHB	BP-1	D2 Glucose	D5 Glycerol
17:30	Base 1	3	x	x	x						
17:45	Base 2	2	x	x							
18:00	1	8	x	x	x	x	x	x	x		
18:15	2	2	x	x							
18:30	3	3	x	x	x						
18:45	4	2	x	x							
19:00	5	8	x	x	x	x	x	x	x		
19:15	6	2	x	x							
19:30	7	3	x	x	x						
19:45	8	2	x	x							
20:00	9	8	x	x	x	x	x	x	x		
20:15	10	2	x	x							
20:30	11	3	x	x	x						
20:45	12	2	x	x							
21:00	13	8	x	x	x	x	x	x	x		
21:15	14	2	x	x							
21:30	15	3	x	x	x						
21:45	16	2	x	x							
22:00	17	8	x	x	x	x	x	x	x		
22:15	18	2	x	x							
22:30	19	3	x	x	x						
22:45	20	2	x	x							
23:00	21	8	x	x	x	x	x	x	x		
23:15	22	2	x	x							
23:30	23	3	x	x	x						
23:45	24	2	x	x							
00:00	25	8	x	x	x	x	x	x	x		
00:15	26	2	x	x							
00:30	27	3	x	x	x						
00:45	28	2	x	x							
01:00	29	8	x	x	x	x	x	x	x		
01:15	30	2	x	x							
01:30	31	3	x	x	x						
01:45	32	2	x	x							
02:00	33	8	x	x	x	x	x	x	x		
02:15	34	2	x	x							
02:30	35	3	x	x	x						
02:45	36	2	x	x							
03:00	37	8	x	x	x	x	x	x	x		
03:15	38	2	x	x							
03:30	39	3	x	x	x						
03:45	40	2	x	x							
04:00	41	10	x	x	x	x	x	x	x	x	x
04:15	42	5	x	x							
04:30	43	5	x	x	x						
04:45	44	5	x	x							
05:00*	45	10	x	x	x	x	x	x	x	x	x
05:15	46	2	x	x							
05:30	47	5	x	x	x						
05:45	48	2	x	x							
06:00	49	10	x	x	x	x	x	x	x	x	x
06:15	50	2	x	x							
06:30	51	5	x	x	x						
06:45	52	2	x	x							
07:00	53	10	x	x	x	x	x	x	x	x	x
07:15	54	2	x	x							
07:30	55	5	x	x	x					x	x
07:35	56	3	x							x	x
07:40	57	3	x							x	x
07:45	58	3	x	x						x	x
07:50	59	3	x							x	x
07:55	60	3	x							x	x

<i>Clock</i>	<i>Sample</i>	<i>Vol</i>	<i>Blood</i>	<i>Growth</i>	<i>Free</i>					<i>D2</i>	<i>D5</i>
<i>Time</i>	<i>No.</i>	<i>ml</i>	<i>glucose</i>	<i>Hormone</i>	<i>Insulin</i>	<i>NEFA</i>	<i>ACA</i>	<i>B-OHB</i>	<i>BP-1</i>	<i>Glucose</i>	<i>Glycerol</i>
08:00	61	10	x	x	x	x	x	x	x	x	x
08:05		0.5	x								
08:10		0.5	x								
08:15	62	2	x	x							
08:20		0.5	x								
08:25		0.5	x								
08:30	63	5	x	x	x					x	x
08:35		0.5	x								
08:40		0.5	x								
08:45	64	2	x	x							
08:50		0.5	x								
08:55		0.5	x								
09:00	65	10	x	x	x	x	x	x	x	x	x
09:05		0.5	x								
09:10		0.5	x								
09:15	66	2	x	x							
09:20		0.5	x								
09:25		0.5	x								
09:30	67	5	x	x	x					x	x
09:35	68	3	x							x	x
09:40	69	3	x							x	x
09:45	70	3	x	x						x	x
09:50	71	3	x							x	x
09:55	72	3	x							x	x
10:00	73	10	x	x	x	x	x	x	x	x	x
10:05		0.5	x								
10:10		0.5	x								
10:15	74	2	x	x							
10:20		0.5	x								
10:25		0.5	x								
10:30	75	5	x	x	x					x	x
10:35		0.5	x								
10:40		0.5	x								
10:45	76	2	x	x							
10:50		0.5	x								
10:55		0.5	x								
11:00	77	10	x	x	x	x	x	x	x	x	x
11:05		0.5	x								
11:10		0.5	x								
11:15	78	2	x	x							
11:20		0.5	x								
11:25		0.5	x								
11:30	79	5	x	x	x					x	x
11:35	80	3	x							x	x
11:40	81	3	x							x	x
11:45	82	3	x	x						x	x
11:50	83	3	x							x	x
11:55	84	3	x							x	x
12:00	85	10	x	x	x	x	x	x	x	x	x
12:05		2	x		x						
12:10		2	x		x						
12:15	86	3	x	x	x					x	x
12:20		2	x		x						
12:25		2	x		x						
12:30	87	5	x	x	x					x	x

### Appendix 3: Computer Program for Hyperinsulinaemic Euglycaemic Clamp

During the hyperinsulinaemic clamp, blood glucose was controlled using a computer programme on laptop computer (PC). The programme is designed to maintain euglycaemia at a level set by the investigator (in this case, all subjects were rendered euglycaemic at 5mmol/l) (Matthews, Edge et al. 1990).

Age, sex, weight and initial blood glucose of the subject is entered into the programme which subsequently calculates dextrose infusion rates based on the blood glucose target. Thereafter, blood glucose concentrations are entered every 5 minutes and the programme accumulates an individual-specific array of blood glucose response to changes in dextrose infusion rate in order to titrated individuals to the euglycaemic target.

## Appendix 4: Western Immunoblotting Methods

### A4.1 Gel Preparation: Solutions

#### *A4.1.1 30% Acrylamide Stock Solution*

100g acrylamide (Sigma A-9099)

2.7g N-N'-methylene-bis-acrylamide (Sigma M-7256)

Add 250ml deionised water to the acrylamide bottle and mix until fully dissolved (approximately 30min).

Add the bis-acrylamide and bring to the final volume with water.

Add approximately 100mg Amberlite MB-1 (Sigma MB1-A), mix briefly and filter through 0.22µm filter using a vacuum filtration unit.

De-gas the solution and store at 4°C in the dark.

#### *A4.1.2 Resolving Buffer (1.5M Tris-base pH 8.8)*

90.85g Tris-base

Add 400ml deionised water

pH to 8.8 with concentrated HCl when the solution is cold

Make up to final volume of 500ml and store at 4°C.

#### *A4.1.3 Stacking Buffer (0.5M Tris-base pH 6.8)*

30.3g Tris-base (Sigma T1503)

Add 400ml deionised water

pH to 6.8 with concentrated HCl when the solution is cold

Make up to final volume of 500ml and store at 4°C.

#### *A4.1.4 Ammonium persulphate (APS, Gibco BRL Cat.No.5523UA)*

10% weight/volume solution to be prepared fresh as needed

250mg APS

2.5ml deionised water

#### *A4.1.5 SDS (Lauryl sulphate) (Sigma L-3771)*

10g SDS (caution, wear a face mask when weighing)

Mix with 100ml deionised water and filter through Whatman 1 qualitative paper

Store at room temperature (may require warming in a water bath to dissolve fully prior to use)



#### *A4.1.6 Gel Apparatus (Hofer Minigel <sup>1</sup>)*

Clean all glass and aluminium plates thoroughly with 70% EtOH.

Grease the gasket slightly with petroleum jelly (do not use too much)

Arrange the sets into the gel holder; order as follows; aluminium plate, spacers, glass plate). Insert as many fillers at the back as required so that the plates do not fall out when the clips are in place.

#### **Pouring Gels**

##### *A4.1.1 Resolving Gel*

Volume Required	4 gels	10 gels
30% Acrylamide	20ml	50ml
Resolving buffer	12ml	30ml
Water	15.1ml	37.8ml
10% SDS	0.48ml	1.2ml
10% APS	0.36ml	0.9ml
TEMED <sup>2</sup>	24µl	60µl

Treat sufficient acrylamide to make both the resolving and stacking gels with amberlite for 10min and filter through Whatman 1 qualitative paper. Mix the acrylamide, resolving buffer and water together and de-gas under vacuum for 10 minutes.

Add the 10% SDS and shake gently (care, this will froth if you are too vigorous), followed by the 10% APS and finally the TEMED.

Once the TEMED has been added, polymerisation will start.

Pour the gel into the apparatus to approximately 0.75cm below the base of the spacer comb. Tap the apparatus firmly to ensure the even distribution of the gel and top up as required.

Mix n-butanol solution (50% water, 50% butanol<sup>3</sup>) and add 300µl to the top of each gel.

Cover the apparatus with parafilm and leave for at least 2 hours at room temperature to polymerise.

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<sup>1</sup> Hofer Mighty Small II vertical slab unit 8 by 7cm gel

<sup>2</sup> N,N,N',N'-tetramethylethylenediamine SIGMA T9281

<sup>3</sup> SIGMA BT 105

#### *A4.1.2 Stacking Gel*

Volume Required	4 gels	10 gels
30% Acrylamide	1.6ml	4ml
Stacking buffer	3ml	7.5ml
Water	7.2ml	18.1ml
10% SDS	0.12ml	0.3ml
10% APS	60µl	150µl
TEMED <sup>4</sup>	7µl	17.5µl

Mix the prepared acrylamide, stacking buffer, water and 10% SDS (there is no need to degas this time).

Pour off the n-butanol from the gels and drain onto blotting tissue, then rinse each gel 3 times with water, drain and blot.

Add the APS and TEMED to the stacking gel solution and mix gently.

Pour the stacking gel solution into the gel apparatus, making sure each gel is filled to the top.

Place the combs into the gels and top up with stacking gel as necessary. Allow to stand at room temperature for 10min, then cover the apparatus with parafilm and leave overnight at 4°C.

Carefully prise the gels apart using a fine spatula and wrap each gel individually in cling-film, marking with the date.

Store in a plastic container at 4°C.

Gels will keep for up to 2 weeks.

#### **SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

##### *A4.1.1 Electrode Buffer*

25mM Tris-base, 192mM glycine<sup>5</sup>, 0.1% SDS, pH 8.3

Prepare 5x concentrated stock solution:

15.1g Tris-base

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<sup>4</sup> N,N,N',N'-tetramethylethylenediamine SIGMA T9281

<sup>5</sup> SIGMA GT 4392

72.1g Glycine

5g SDS

Add deionised water to 800ml, pH to 8.3, then add water to final volume 1000ml. Store at room temperature.

#### *A4.1.2 Loading Buffer (4x concentrated stock solution)*

13.3% SDS, 0.42M Tris-base, 0.013% bromophenol blue<sup>6</sup>, pH 6.5

6.65g SDS

3.31g Tris-base

Add water to 50ml and pH to 6.5

Finally add 6.5mg bromophenol blue

#### *A4.1.3 Sample Preparation*

Serum Samples: Prepare 1x sample buffer, by mixing 3ml water, 0.6ml loading buffer and 0.4ml glycerol<sup>7</sup>. Mix 5µl serum with 45µl sample buffer and vortex for 5 seconds

Urine Samples: Prepare 4x sample buffer, by mixing 0.6ml loading buffer and 0.4ml glycerol. Mix 37.5µl 10X concentrated urine with 12.5µl 4x sample buffer and vortex for 5 seconds.

Molecular Weight Rainbow Markers<sup>8</sup>: Add 5µl markers to 15µl 1x sample buffer and vortex for 5 seconds (Hossenlopp, Seurin et al. 1986).

Denature all samples at 60°C for 10 minutes.

#### **Loading and running the gel**

Grease the gaskets and place in position. Check the gel for bubbles and that the stacking gel is right to the top, then place it in position and secure it with the clips.

Fill the bottom tray almost to the top with 1x electrode buffer and then fill the top chamber with the same to completely cover the gels.

Remove the comb carefully from the gel and top up as required.

Allow the denatured samples to cool slightly before loading onto the gel (markers; 10µl per lane, samples 25µl per lane).

Run at 25 to 30mA per gel (200V) for approximately 2 hours or until the dye front nears the bottom of the gel<sup>9</sup>.

#### **Membrane Transfer**

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<sup>6</sup> SIGMA molecular biology reagent B5525 = 3',3'',5',5''- tetrabromophenolsulphonaphthalein

<sup>7</sup> SIGMA G7893

<sup>8</sup> Amersham RPN 756

<sup>9</sup> Pharmacia LKB-EPS 500/400 power source.

#### *A4.1.1 Transfer Buffer (10x concentrated stock solution)*

15mM Tris-base, 120mM glycine, 20% methanol, pH 8.3

18.17g Tris-base

90.08g glycine

Add water to 1000ml.

To make a 1x solution, mix 10ml stock solution with 20ml methanol<sup>10</sup> and 70ml water.

Adjust to pH 8.3 before using.

#### *A4.1.2 Membrane Preparation*

Cut Whatman 3mm chromatography/ filter paper to 6.5 by 8.5cm (8 pieces per gel).

Cut 1 piece of Hybond-C extra transfer membrane<sup>11</sup> per gel to 6.5 by 8.5cm (do not handle directly, wear gloves or use forceps).

#### *A4.1.3 Methods*

Turn off the power supply and remove the gel from the apparatus.

Carefully prise off the glass plate from the gel.

Cut off the stacking gel and mark the top right corner of the gel by cutting the corner off diagonally. Remove the gel from the aluminium plate and place into a tray with 1x transfer buffer.

Wet 4 filter papers in 1x transfer buffer and place onto the base of the semi-dry electroblotter<sup>12</sup>.

Wet the transfer membrane in 1x transfer buffer and place carefully onto the filter papers.

Place the gel onto the membrane and carefully smooth out any air bubbles.

Finally add a further 4 filter papers wetted in 1x transfer buffer.

Run the electroblotter at 27mA per gel (200V) for 90min.

Air dry the membrane onto blotting paper and mark the position of the molecular weight markers. Store at room temperature.

#### *Western Immunoblotting (Chemiluminescence method)*

##### *A4.1.1 PBS-T20 (Phosphate buffered saline, Tween 20)*

11.5g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O (Molecular weight 177.99)

2.96g NaH<sub>2</sub>PO<sub>4</sub> (Molecular weight 119.98)

5.84g NaCl

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<sup>10</sup> Rathburn Chemicals Ltd, Walkerburn, Scotland Cat # RH1019

<sup>11</sup> Amersham RPN.303E

<sup>12</sup> Semi-Phor, Hoefer, Inc.

Dissolve into 800ml deionised water and adjust the pH to 7.5. Make up volume to 1000ml with water and finally add 1ml Tween-20.

#### *A4.1.2 10% Milk Solution*

Dissolve 5g skimmed milk powder in 50ml PBS-T20

#### *A4.1.3 3% Bovine Serum Albumin*

Add 1.5g of BSA <sup>13</sup>(fraction V) to 50ml PBS-T20. Do not mix or stir, but allow to stand at room temperature until it has dissolved.

#### *A4.1.4 1% Bovine Serum Albumin*

Add 0.5g BSA (fraction V) to 50ml PBS-T20. Do not mix or stir, but allow to stand at room temperature until it has dissolved.

#### *A4.1.5 Immunoblotting the membrane*

All washes and antibody incubations should be performed whilst shaking gently.

Take the dried membrane and block in 10% milk solution for 60min at room temperature.

Transfer the membrane into 3%BSA solution and block for a further 30min at room temperature.

Rinse the membrane briefly with PBS-T20, then wash in PBS-T20 for 15min at room temperature.

Repeat a further 2 5min washes in PBS-T20.

Prepare the primary antibody (anti IGFBP-3<sup>14</sup>) 1 in 1000 dilution in 1% BSA solution, 5ml per membrane.

Seal membrane and primary antibody solution into a bag and incubate overnight at 4°C.

Remove the membrane from the bag and rinse briefly with PBS-T20.

Wash the membrane once at room temperature for 15min in PBS-T20.

Repeat at least a further 2, 5-min washes in PBS-T20.

Prepare the secondary antibody (1 in 8000 dilution<sup>15</sup>) in PBS-T20, 5ml per membrane.

Seal membrane and secondary antibody solution into a bag and incubate at room temperature for 60min.

Remove the membrane from the bag and rinse briefly with PBS-T20.

Wash the membrane once at room temperature for 15min in PBS-T20.

Repeat at least a further 2, 5-min washes in PBS-T20.

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<sup>13</sup> Albumin Bovine Serum, Fraction V, Sigma A9647

<sup>14</sup> Upstate Biotechnology Inc, Lake Placid, NY, USA Cat. # 06-108

<sup>15</sup> Anti-rabbit IgG, horseradish peroxidase, Amersham Irl Plc, Bucks, UK. Cat. # NA-934

Once the washes are completed, keep the membrane in the final wash until you are ready to perform the chemiluminescent detection.

#### *A4.1.6 Chemiluminescent Detection*

This should be performed in the dark room using a commercially available enhanced chemiluminescent (ECL) detection kit<sup>16</sup>.

Mix 5ml ECL solution 1 with 5ml ECL solution 2 and pour onto the still wet membrane in a large weigh boat.

Shake gently by hand for 60 seconds, then remove the membrane and dry briefly on filter paper, before wrapping the membrane in cling film.

Expose to X ray film for 45 seconds, then develop the film and re-expose if required.

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<sup>16</sup> Enhanced Chemiluminescent Detection System, Amersham Intl Plc, Bucks, UK Cat. # RPN 2109



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## Publications Arising From This Thesis

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- 1) **Williams, R.M.**, K. Yuen, D. White, et al., *Short-term suppression of elevated growth hormone concentrations following insulin-like growth factor 1 administration in young adults with type 1 diabetes does not alter glomerular filtration or albumin excretion rates*. Clin Endocrinol (Oxf), 2006. **65**(4): p. 439-45.
- 2) **Williams, R.M.**, R. Amin, F. Shojaee-Moradie, et al., *The effects of a specific growth hormone antagonist on overnight insulin requirements and insulin sensitivity in young adults with Type 1 diabetes mellitus*. Diabetologia, 2003. **46**(9): p. 1203-10.